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ABSTRACT

Presented are laboratory studies focusing on blood cells and the complete scheme of blood coagulation. Formed is the basis for the following types of laboratory operations: (1) distinguishing the morphology of normal and abnormal blood cells; (2) measuring the concentrations or number of blood cells; (3) measuring concentration and detecting abnormalities of hemoglobin; (4) measuring defects in coagulation; and (5) performing a few specific disease-related tests which involve blood cells. Types of equipment needed, actual stepwise performance of tests, and reagents needed, as well as established minimum levels of accuracy are included. (Author/CS)

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DEPARTMENTS OF THE AIR FORCE
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AF MANUAL 160–51
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Medical Service

CLINICAL LABORATORY PROCEDURES—HEMATOLOGY

The purpose of this manual is to present the laboratory studies which concern the blood cells and the complete scheme of blood coagulation. It forms the basis for the following types of laboratory operations: (1) distinguishing the morphology of normal and abnormal blood cells; (2) measuring the concentrations or number of blood cells; (3) measuring concentration and detecting abnormalities of hemoglobin; (4) measuring defects in coagulation; and (5) performing a few specific disease-related tests which involve blood cells. It enumerates types of equipment needed, actual stepwise performance of tests, and reagents needed, as well as establishes minimum levels of accuracy.

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Chapter 1

BLOOD

SECTION A—THE COMPOSITION OF BLOOD

1-1. Introduction. Blood is a complex and unique fluid of variable composition circulating through the vascular system of the body. It is a tissue in which cellular constituents are suspended in a liquid medium performing specialized functions. The prime function of blood is to maintain oxygen and food supply for the body cells and prevent accumulation of waste products. Blood also protects against causative agents of disease and supplies substances which promote the clotting process when injury causes bleeding or hemorrhage. To perform these complex functions, the composition of blood must be complex. Formed elements account for approximately 45 percent of the blood volume. The remaining 55 percent is a liquid of a very complex nature called plasma. Plasma is composed mostly of water but it also contains other important substances.

1-2. General Cellular Structure:

a. A typical cell is composed of a single nucleus embedded in cytoplasm. The living substance of the cell is a grayish viscous liquid called protoplasm. Protoplasm is enclosed in the cell interfaces by a cell membrane which selectively regulates the interchange of materials between the cell and its environment. A typical cell is diagramed in figure 1-1.

b. The nucleus is a spherical or oval body surrounded by a thin membrane (nuclear membrane). Contained in the nucleus is a sphere called the nucleolus. The nucleolus is thought to be an organizing center for the cell and can have the capacity for cell production. The absence of nucleoli signifies the end of cell development. Also found within the nucleus is a network of nuclear fibrils made up of DNA (deoxyribonucleic acid) and protein called chromatin. It is thought that the decreasing growth activity of a cell during maturation is regulated by chromatin. A typical cell nucleus is diagramed in figure 1-1.

c. Surrounding the nucleus is a mass of protoplasm called cytoplasm. Contained within the cytoplasm are numerous granules, filaments, and globules. These structures are divided into two groups known as organoids (organelles) and inclusions. The organoids are thought to perform most of the metabolic functions of the cell. Mitochondria, Golgi apparatus, fibrils, centrioles, and the chromatin substance are classified as organelles. Cytoplasm inclusions are usually seen as granulation. This granulation is an accumulation of proteins, lipids, carbohydrates, pigments, and secretory granules.

1-3. Cellular Constituents:

a. Erythrocytes. An erythrocyte (red blood cell) is an elastic, nonnucleated, biconcave disc having a diameter of approximately 7.2 microns. The mature red cell contains about 34 percent hemoglobin (a complex iron-bearing pigment which transports oxygen). Hemoglobin is contained in the interior of the cell, and the outer surface of the cell is surrounded by a cell membrane. When unstained, the cell has a pale, greenish-yellow appearance; and light orange with an accented central zone of pallor when stained with Wright's stain. The production of erythrocytes, or erythropoiesis, occurs primarily in the red marrow of the spongy bones (see figure 1-2). An adult female has approximately 4.8 million/cu mm red cells, and an adult male has approximately 5.4 million/

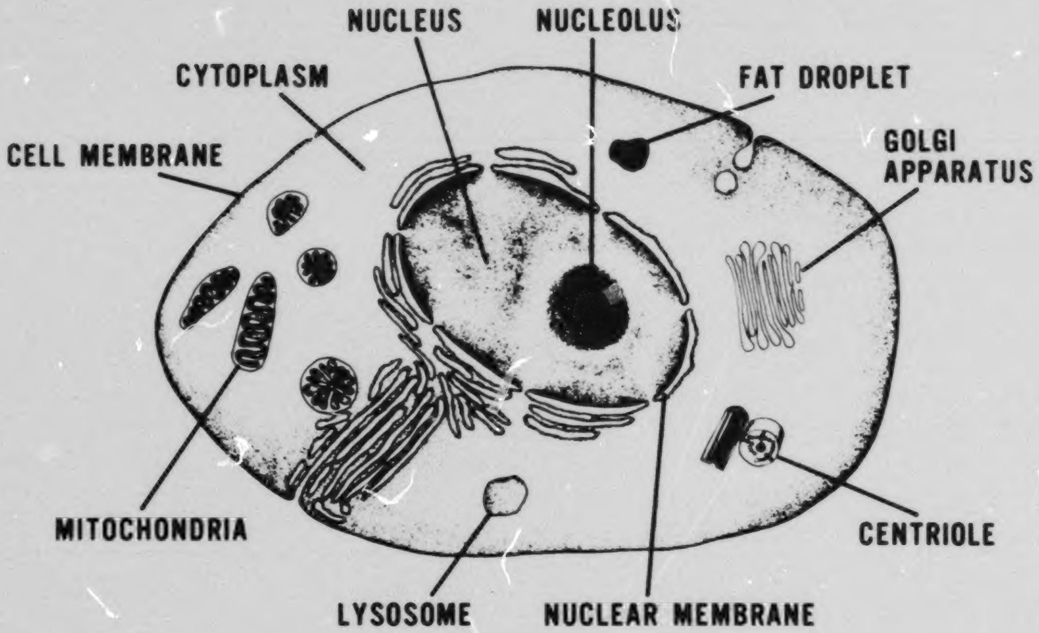


Figure 1-1. Typical Cell Structure.

cu mm red cells. The erythrocytes have an average life span of 80 to 120 days.

b. Leukocytes. Leukocytes are commonly known as white blood cells because of their lack of color in unstained preparations. They are nucleated cells which have an average diameter of 8 to 12 microns. Leukocytes are divided into three groups: (1) granulocytes, (2) lymphocytes, and (3) monocytes. They are differentiated by the specific nuclear and cytoplasmic staining properties.

c. Thrombocytes. Thrombocytes are most commonly known as platelets. Platelets are detached fragments of the cytoplasm of their precursor cells—megakaryocytes which are found in the bone marrow. Unstained platelets appear as small hyaline structures with a diameter of approximately 2 microns. When stained with Wright's stain, they have a pale blue cytoplasm with a dark granular center. Platelets are very fragile and live for a period of about 3 to 5 days.

1-4. Liquid Constituents:

a. Plasma. Blood plasma is the fluid portion of the blood before clotting occurs (serum plus fibrinogen). The chemical nature of plasma is very complex. Plasma is composed mostly of water but it also contains dissolved gases (oxygen, carbon dioxide, and nitrogen), proteins (albumin, globulin, and fibrinogen), carbohydrates (glucose), lipids (fats, lecithin, and cholesterol), nonprotein nitrogenous substances, and inorganic salts.

b. Serum. Serum is the fluid portion of blood after the clotting process is complete. Fibrinogen, the precursor of fibrin, is removed from plasma to form the framework of the blood clot.

SECTION B—FORMATION OF BLOOD CELLS

1-5. Embryonic Hematopoiesis:

a. The primary source of blood cells is the mesenchyme connective tissue in the embryo. Three phases of embryonic hematopoiesis merge, resulting in the formation of blood.

b. During the first 2 months of embryonic development the mesoblastic phase occurs. The blood cells are formed in the blood islands of the yolk sac. Immature blood cells develop, having large nuclei containing a vesicular chromatin meshwork. The nuclei are surrounded by a thin rim of cytoplasm. The cells gradually develop hemoglobin to become a nucleated red blood cell. Mitosis occurs and daughter cells contain more hemoglobin. Finally the nucleus is lost and the mature erythrocyte is produced.

c. At 2 months the hepatic phase begins. Blood cell development shifts to the body of the fetus as the organs of the reticuloendothelial system (liver, spleen, thymus, etc.) are formed. During this phase, red cells begin their normal development. Granulocytes and thrombocytes are formed in the liver while lymphocytes and monocytes are formed in the spleen and thymus.

d. The myeloid phase begins during the fifth month of gestation. Blood cells are formed in the bone marrow and lymphatic system which at the time of birth constitute the total sources of hematopoiesis. The bone marrow is the principle source of production of erythrocytes, granulocytes, and thrombocytes. The lymph nodes are the primary sites of production of lymphocytes, monocytes, and plasmocytes.

1-6. Postnatal Hematopoiesis:

a. Blood formation at birth is confined primarily to the bone marrow (central medullary structure of the bone). Blood cells multiply by mitosis and then mature to a specific cell type. The mature cells lose the ability to reproduce and develop a definite life span. Regeneration of blood cells after birth involve multiplication of precursor cells, evolution of the definitive characteristics of each type, and release of mature cells.

b. Myelopoiesis is the production of blood cells by the bone marrow (medullary site of production). The red bone marrow is the principle source of production of red cells and white cells of the granulocytic series. At birth the central medullary structure of bones is red bone marrow and it is actively engaged in hematopoiesis. At about 5 years of age a nonhematopoietic type of marrow (fatty yellow bone marrow) which is a reserve potential tends to replace most of the red bone marrow. This partial replacement of red marrow is complete when the individual reaches maturity (about 18 years of age) at which time active hematopoietic centers in bone tissue are limited almost exclusively to the sternum, pelvic area, vertebrae, skull, ribs, clavicles, scapuli, and the epiphyses of the long bones (figure 1-2).

c. Extramedullary hematopoiesis is blood production which occurs in sites other than the bone marrow. Active sites are the spleen, thymus, lymph nodes, and other lymphoid tissues (figure 1-2). Cell production is largely limited to lymphopoiesis. The lymph nodes are the primary source of lymphocytes and plasmocytes.

SECTION C—NORMAL CELL MATURATION

1-7. General Features. In the course of blood cell maturation, certain specific features are developed. Each of the component parts of the cell undergoes a transformation during maturation. The immature cell or blast cell contains a large nucleus, a small amount of cytoplasm, and no granules. As the cell ages, the cytoplasm becomes less basophilic and nuclear chromatin becomes heavier (darker stain). Reduction in size and loss of nucleoli occurs as the cell becomes older. The three types of granulation (neutrophilic, basophilic, and eosinophilic) become more specific and smaller as the cell ages. Maturation, in general, involves: (1) cytoplasmic differentiation, (2) nuclear maturation, and (3) reduction in cell size (see figure 1-3).

1-8. Cytoplasm. The basophilia of a blast cell is proportional to the ribonucleic acid (RNA) content. As the cell matures the RNA content decreases and the cell becomes a paler blue. In the myeloid cells a specific

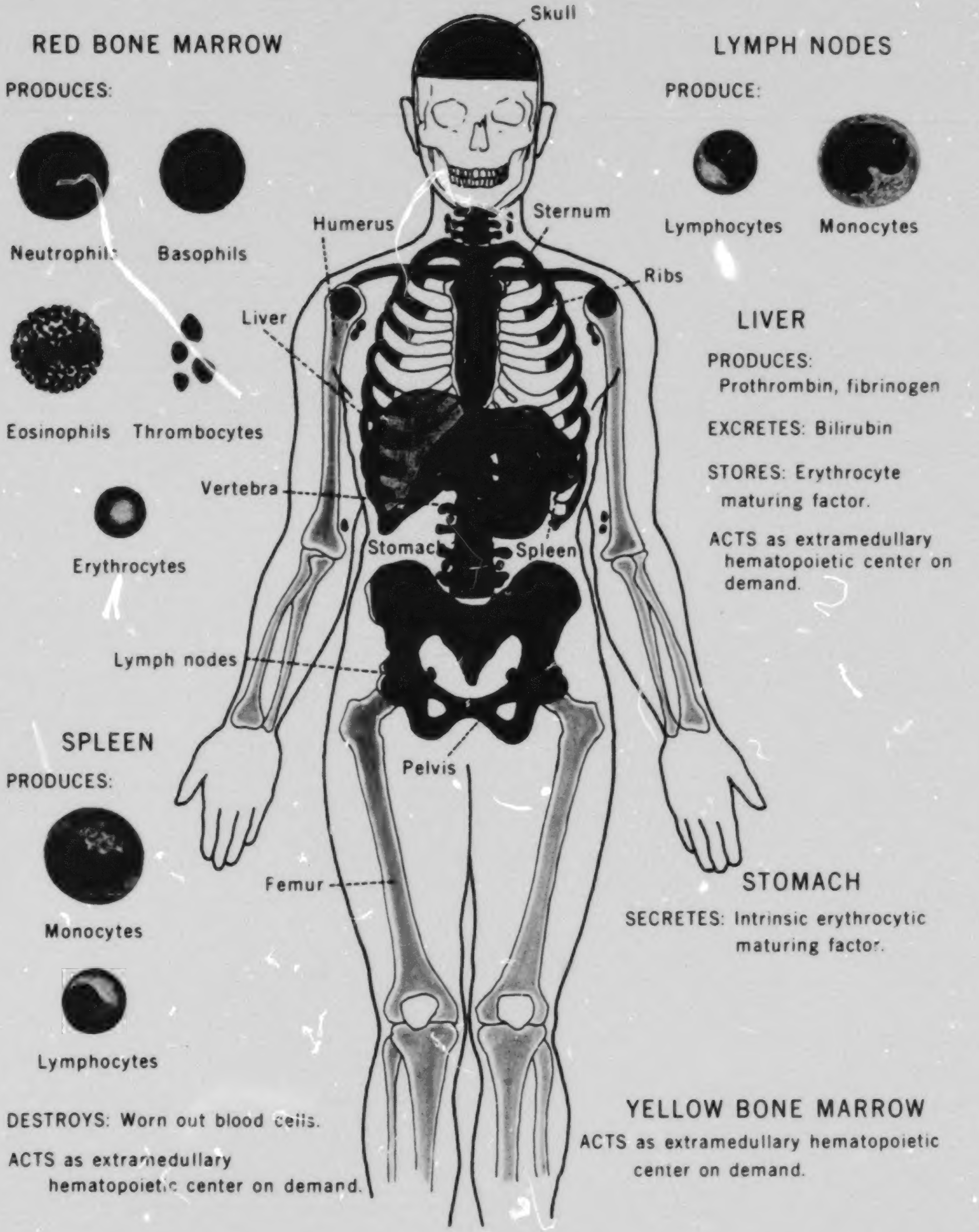

RED BONE MARROW
PRODUCES:
Neutrophils
Basophils
Eosinophils
Thrombocytes
Erythrocytes
Skull
Humerus
Sternum
Ribs
Liver
Vertebra
Stomach
Spleen
Lymph nodes
Pelvis
Femur
LYMPH NODES
PRODUCE:
Lymphocytes
Monocytes
LIVER
PRODUCES: Prothrombin, fibrinogen
EXCRETES: Bilirubin
STORES: Erythrocyte maturing factor.
ACTS as extramedullary hematopoietic center on demand.
SPLEEN
PRODUCES:
Monocytes
Lymphocytes
DESTROYS: Worn out blood cells.
ACTS as extramedullary hematopoietic center on demand.
STOMACH
SECRETES: Intrinsic erythrocytic maturing factor.
YELLOW BONE MARROW
ACTS as extramedullary hematopoietic center on demand.

Figure 1-2. Hematopoietic System in an Adult 18-20 Years of Age.

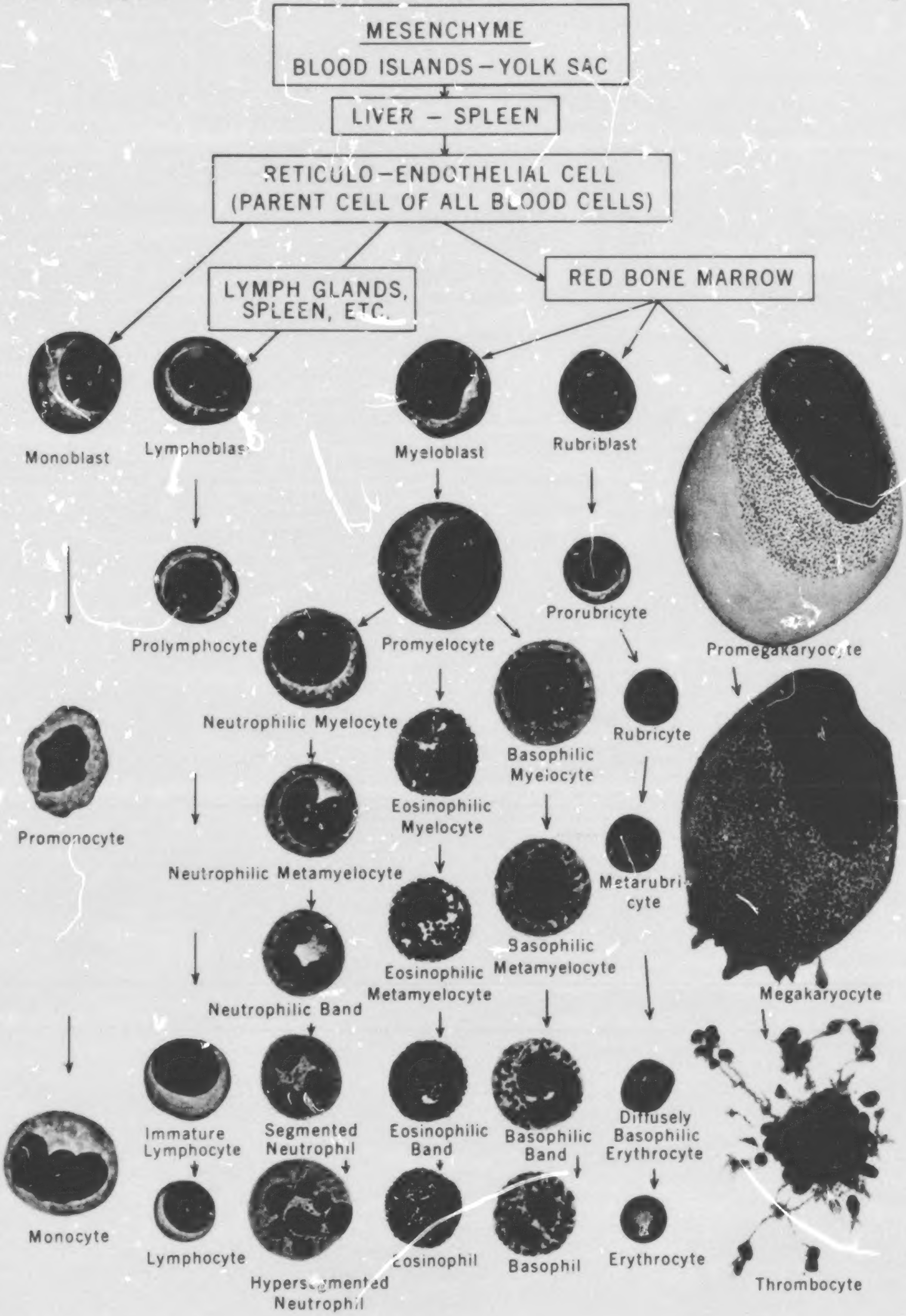

MESENCHYME
BLOOD ISLANDS—YOLK SAC
LIVER — SPLEEN
RETICULO—ENDOTHELIAL CELL
(PARENT CELL OF ALL BLOOD CELLS)
LYMPH GLANDS, SPLEEN, ETC.
RED BONE MARROW
Monoblast
Lymphoblast
Myeloblast
Rubriblast
Prolymphocyte
Promyelocyte
Prorubricyte
Promegakaryocyte
Neutrophilic Myelocyte
Rubricyte
Basophilic Myelocyte
Promonocyte
Eosinophilic Myelocyte
Neutrophilic Metamyelocyte
Metarubricyte
Basophilic Metamyelocyte
Eosinophilic Metamyelocyte
Neutrophilic Band
Megakaryocyte
Immature Lymphocyte
Segmented Neutrophil
Eosinophilic Band
Basophilic Band
Diffusely Basophilic Erythrocyte
Monocyte
Lymphocyte
Hypersegmented Neutrophil
Eosinophil
Basophil
Erythrocyte
Thrombocyte

Figure 1-3. Development of Blood Cells.

type of granulation occurs. When granules appear they are pinkish red and few in number. The granules increase in number and differentiate into three types upon maturation. As the cell matures it develops an affinity for the acid or basic portion of the stain (Wright's stain). Basophilic granules stain blue, eosinophilic granules stain bright orange, and neutrophilic granules stain pinkish-purple. Lymphocytes are usually devoid of cytoplasmic granulation but they can possess nonspecific azurophilic (dark purple) granules, usually characteristic of monocytes and plasmocytes. Upon maturation, the erythrocyte develops a light orange respiratory pigment called hemoglobin.

1-9. Nucleus. The nucleus of the young cell is large, round, and occupies most of the cell. As the cell matures the size of the nucleus decreases. Nuclei of early or primitive cells usually have one or more nucleoli. The latter are small, round, homogeneous areas which usually stain light blue with a darker boundary. In appearance nucleoli are somewhat like craters in the nucleus. They are surrounded by strands of chromatin. These nucleoli, plus a delicate reticular network of chromatin, are the principal indicators of bood cell immaturity. As the cells mature the nucleus gradually becomes smaller, stains darker, and the chromatin meshwork become "coarse" with the strands of chromatin less fine and lacelike. In the course of cell development the nucleus changes its shape, particularly in the granulocytic series, where it becomes indented, lobulated, segments, or fragmented. As maturation or development progresses, the nucleus, if still intact, becomes small, compact, usually dark, and structureless and can completely disappear. The loss or shrinking of the nucleus is accompanied by a decrease in cell size.

SECTION D—ABNORMAL CELL MATURATION

1-10. General Features. Abnormal cell maturation is asynchronous development as opposed to normal cell maturation or synchronous development. Since the normal sequence of cell maturation is upset, atypical cells will be present. Abnormal cells can be recognized by: (1) abnormal cytoplasmic maturation, (2) abnormal nuclear maturation, and (3) abnormal size.

1-11. Cytoplasm. Asynchronism of the cytoplasm is most commonly seen in the granulocytes. Granulation can be primitive or absent. In some instances the granules fail to differentiate. Erythrocytes show basophilia late in the series and retarded hemoglobination. Inclusions, in the cytoplasm, such as Dohle bodies (infectious diseases), Auer rods (leukemia), and toxic granulation (infection affecting the marrow) are seen in the abnormal white cells.

1-12. Nucleus. Abnormal cells often show two nuclei in severe disturbances, such as leukemia. Nucleoli have a retarded reduction. The nucleus can have an irregular outline or indentation (Rieder cells). Hypersegmented nuclei occur in the neutrophils in sepsis and in pernicious anemia. Abnormal maturation of the nucleus often results in variation in cell size.

SECTION E—FUNCTIONS OF BLOOD CELLS

1-13. Introduction. Each component of blood is uniquely capable of performing one or more functions. Together, these components provide the maintenance of a relatively stable environment of the body by a variety of mechanisms. This maintenance of a relative biological constancy or integrity is known as homeostasis. Once the blood cells reach full maturity they enter the bloodstream and begin fulfilling their functions.

1-14. Erythrocytes. Hemoglobin is the main functioning component of the cell. It carries out the transportation of oxygen to the tissues and the removal of carbon dioxide. Hemoglobin also aids in the maintenance of the delicate acid-base buffer system of the body. The erythrocyte must also supply energy to accomplish the active transport of glucose and ions against a gradient across the red cell membrane.

1-15. Leukocytes. Leukocytes remove invading antigens (for example, bacteria) and to some extent transport and distribute antibodies. Monocytes and all of the granulocytes have been shown to demonstrate directional movement. Their movement is subject to chemotaxis, which is defined as the response of living protoplasm to a chemical stimulus. This is a means of attracting cells to substances which they must either transport or engulf. The process of engulfing and destroying bacteria, or phagocytosis, is a prime function of leukocytes.

a. Monocytes. These cells will engulf bacteria and larger materials, including even protozoa and red cells, and are called macrophages. In this regard, monocytes are perhaps the most efficient phagocytes of all the cells. Monocytes contain many of the lytic enzymes that are found in microphages (granulocytes). In addition, monocytes contain lipases which enable them to dissolve the lipoid capsules of certain bacteria.

b. Neutrophils. Neutrophilic leukocytes are excellent microphages. That is, they engulf bacteria and other microscopic particles. The particles are first surrounded by cellular pseudopodia and then incorporated into a cell vacuole. There the foreign bodies mix with substances released from the cytoplasm of leukocytes. In this way the leukocyte is not injured by whatever "combat activity" is taking place in the vacuole. Neutrophils are fully-developed (mature) cells that are incapable of mitotic division. They carry on active metabolism. Eventually the granulocytes disintegrate and in inflammatory processes are succeeded by monocytes.

c. Eosinophils. Eosinophils are found in tissue fluid as well as in peripheral blood, especially in areas where there is an allergic reaction. Current thinking holds that eosinophils are involved in antigen-antibody reactions, and have been shown to phagocytize antigen-antibody reactants. Eosinophils are also thought to transport, or at least contain, lysins which act on fibrin. It is suggested that eosinophils limit the action of substances such as histamine. How this is accomplished is not yet clear. The mobilization of eosinophils from their reserve in the bone marrow is at least in part under hormonal control. If the adrenal cortex is functioning properly, an injection of adrenocorticotropic hormone (ACTH) results in a marked decrease in the number of circulating eosinophils and in the number of circulating lymphocytes. On the other hand, there is an increase in the number of circulating neutrophils.

d. Basophils. The function of basophils in man has not been ascertained. They quite possibly represent a vestige of evolution. Their granules have been found to contain heparin, and these cells frequently appear during the clot dissolution phase of an injury. Hence, it has been suggested they may be involved in clot absorption.

e. Lymphocytes. The lymphocyte is now believed to be directly connected with antibody production. Undoubtedly, the lymphocyte performs important immunologic functions. According to very recent studies, many of the activities previously thought to take place in the reticuloendothelial (RE) system actually take place in lymphocytic tissue.

1-16. Platelets. Platelets possess metabolic systems, expend energy, and respond to stimuli. They contain many enzymes and undergo respiratory activity and glycolysis. They possess coagulation factors usually designated as PF-1, PF-2, and on through PF-7. The cells contain fibrinogen and vasoconstrictor substances, calcium, and many other components which are either known or presumed to participate in the clotting mechanism. Clot-promoting lipoproteins are also found in platelets. In addition, well-defined antigens have been found in platelets. The role of platelets in the blood coagulation mechanism will be described in more detail in chapter 6.

Chapter 2

MATERIAL EMPLOYED IN HEMATOLOGY

SECTION A—LABORATORY REAGENTS

2-1. Preparation. Various stains and solutions are utilized in routine hematological examinations. These stains and solutions must be prepared with the utmost care and precisely according to formulations. Detailed directions for the preparation of all reagents which are required for performing procedures outlined throughout this manual are contained in the respective procedure. Careful attention should be given to precise measurements, order in which reagents are added, control of temperature where indicated, filtration, and aging. Particular attention must be given to storage of reagents particularly with reference to requirements for refrigeration, incubation, and protection from intense light.

2-2. Labeling Reagent Containers. Proper labeling of reagents is an extremely important detail. Labels should be complete, securely attached, and neatly and legibly written or preferably typewritten. Items recorded on the label should include all constituents and quantities utilized, date of preparation, initials of the individual who prepared the reagent, and expiration date if the solution deteriorates with age. Labels should be protected against damage from water or other fluids by covering with a protective coating of cellophane tape over the surface of the label.

2-3. Safety Precautions. There are various precautions which must be taken in handling reagents in the hematology laboratory. Among the most important are the following:

a. Once a portion of a reagent has been removed from the original container, it should never be poured back because it can contaminate the remaining reagent.

b. Reagents are preferably stored in alphabetical order on shelving protected from dust, moisture, and direct sunlight.

c. Never use a reagent which cannot be clearly identified from the label on the container. Discard all reagents which cannot be accurately identified.

d. Always read the label before dispensing a reagent.

e. When working with newly prepared reagents, especially stains, ascertain whether desired results are being obtained. Unsatisfactory solutions should be discarded and replaced.

f. All mixing containers, stirring rods, and containers used for storage of reagents should be chemically cleaned prior to use.

g. During mixing and preparation, as well as in storage, it is good practice to avoid contact of reagents with metals. Many reagents contain substances which will react chemically with metals and produce changes which will render them unusable for laboratory work.

h. Do not allow inexperienced personnel to prepare reagents without close supervision.

i. Certain reagents are poisonous (that is, Drabkin's solution and bichloride of mercury), and adequate precautions should be taken to prevent accidental poisoning. All highly toxic reagents should be conspicuously labeled "POISON" and should be stored in a separate cabinet in the laboratory.

j. Commercial reagents should be checked with standards for purity. Record all lot numbers in case a reagent is not pure.

k. Test all new reagents to assure that proper results are attainable.

SECTION B—LABORATORY GLASSWARE

2-4. Diluting Pipets:

a. The blood cell diluting pipet (see figure 2-1) consists of a graduated capillary tube having an arbitrary volume of one unit and marked in increments of that unit, each designated as 0.1; note that this unit is not a standard measurement but merely an arbitrarily selected unit. Above the capillary tube is a mixing bulb containing a color-coded glass bead, and above the bulb another shorter capillary tube with an engraved mark. The pipet for performing the white blood cell count has a white bead, the mixing bulb is smaller than that of the red count pipet, and the marking above the bulb reads "11." The pipet for performing the red blood cell count has a red bead in the mixing bulb and the marking above the bulb is "101."

b. These pipets are used to take the specimen directly from a capillary puncture or, after careful mixing, from a vial of fluid or of blood treated with an anticoagulant, such as EDTA. The blood or fluid is drawn into the pipet to a predetermined point and diluted to the correct mark with diluting fluid. After proper mixing, the diluted substance is placed in the counting chamber and the cells are counted.

c. Usually, the technique for diluting the blood specimen with a pipet calls for whole blood to be drawn exactly to the 0.5 mark and diluted only to the "11" or "101" mark with appropriate diluting fluid dependent upon the type of cell count. Since the volume of fluid in the stem does not enter into the dilution, the dilution is calculated on the volume in the bulb, thus with the white blood cell count:

$$\text{Dilution} = \frac{\text{Whole blood}}{\text{Volume in bulb}} = \frac{0.5}{10} = \frac{1}{20}$$

and in the RBC count

$$\text{Dilution} = \frac{\text{Whole blood}}{\text{Volume in bulb}} = \frac{0.5}{100} = \frac{1}{200}$$

The dilution of the blood sample in the white blood count is 1 to 20 and the dilution factor is 20. In the red blood count the blood sample is diluted 1 to 200 and the dilution factor is 200. The permissible error of the red cell pipet is ±5% and the white cell pipet has a permissible error of ±3.5%.

d. Blood cell diluting pipets are delicate pieces of equipment and should have careful treatment. It is also important that clean, dry diluting pipets be used to prevent dilution errors and hemolysis of cells.

e. If an aspirator is available, pipets can be easily washed by drawing cold tap water (distilled water is preferred) through the pipet. If a deposit remains, it can be dissolved by drawing dilute sodium hypochlorite (household bleach) through the pipet fol-

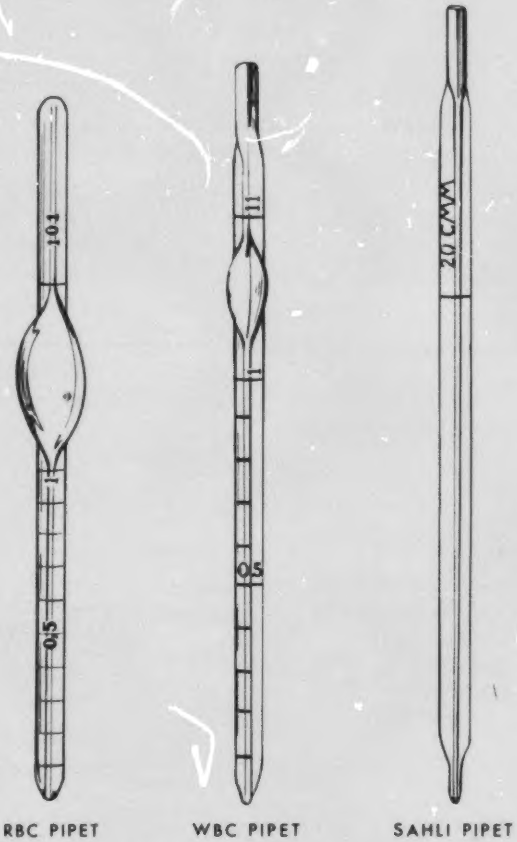


Figure 2-1. Hematological Pipets.

lowed by several washings with distilled water. If pipets become plugged through neglect, the hole may be opened with a fine steel wire, and the above procedures may then be carried out. When necessary, pipets may be dried rapidly by drawing a small amount of alcohol through them and then ether or acetone followed by a stream of air until the glass bead moves freely and no moisture remains in the pipet.

f. All pipets should be inspected each time before use so as to prevent the use of equipment that is dirty or that has chipped ends.

2-5. Unopettes:

a. The Unopette system (Becton-Dickinson and Co.) consists of a disposable uniform-bore glass capillary pipet with an attached plastic tab for handling. The pipet (see figure 2-2) is attached to a plastic reservoir in which predetermined amounts of diluting fluids, depending on their purpose, can be placed. The blood aspirated into the diluting fluid can be mixed and dispensed with great ease and speed.

b. The Unopette system can be used for a variety of hematological procedures. In general, the Unopette system is used as follows:

(1) Using the pointed end of protective plastic shield of the capillary holder, apply firm and even pressure and puncture seal in top of plastic reservoir.

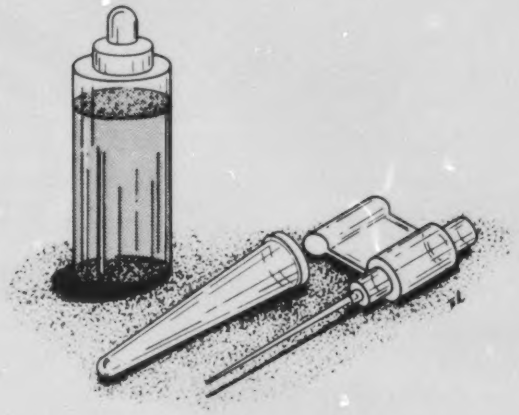


Figure 2-2. Unopette System.

(2) Remove protective plastic shield covering capillary. Obtain blood sample from free-flowing finger puncture (or use thoroughly mixed venous blood specimen).

(3) Holding capillary almost horizontal, touch tip to blood. Capillary action fills the pipet. Carefully wipe excess blood off the tip without removing any blood from the inside of the tube.

(4) Squeeze reservoir slightly. Cover opening of overflow chamber with index finger and seat holder in reservoir neck. Release pressure on reservoir and remove finger from overflow chamber opening. Suction will draw blood into diluent.

(5) Squeeze reservoir gently two or three times to rinse capillary tube, forcing diluent up into, but not out of, overflow chamber, releasing pressure each time to return diluent to reservoir. Mix blood sample with diluent by inversion.

(6) To dispense, reverse capillary tube, seal overflow chamber end onto reservoir, invert and squeeze. Procedures for the Unopette system are available in the manufacturer's instruction booklet.

(7) Always follow manufacturer's instructions.

2-6. Calibration of Sahli Pipets:

a. Introduction. Sahli hemoglobin pipets are volumetric pipets (see figure 2-1). The volume is "supposed" to be 20 lambda as registered on the side of the pipet. Normally, because this volume is not an accurate measurement, standardization of the pipet is necessary. This is usually done with mercury and the procedure is necessary to ascertain the correction factor for each pipet.

b. Procedure:

(1) Heavily lubricate the barrel of a 2 ml syringe with stopcock grease.

(2) Tightly fit the tip of the syringe into the broad end of a 1-holed rubber stopper, size 0.

(3) Clamp the stopper to a heavy retort stand. Insert the base of the pipet into

the other end of the rubber stopper until it is in contact with the tip of the syringe. Adjust the assembly so that the pipet occupies a vertical position.

(4) Allow the mercury and the pipet to reach room temperature before starting the calibration.

(5) Determine the temperature of the mercury.

(6) Slightly withdraw the plunger of the syringe.

(7) Hold the beaker containing mercury so that the tip of the pipet is immersed.

(8) Withdraw the plunger of the syringe until mercury reaches the 20 cu mm mark.

(9) Hold the barrel of the syringe steady and quickly pull the beaker of mercury away from the tip of the pipet. (Slight fluctuation in the column of mercury at this point has no appreciable significance provided there is no loss of mercury from the tip of the pipet.)

(10) Expel the mercury from the pipet into a weighing bottle by manipulation of the syringe. (The weight of the weighing bottle is precisely determined before starting the calibration.)

(11) Weigh the mercury and the weighing bottle on an analytical balance.

c. Calculations:

(1) Determine the weight of the pipetted mercury by subtraction: (weight of weighing bottle + mercury) − (weight of weighing bottle). As an example consider the weight of the mercury to be 0.268 grams.

(2) Convert grams to milligrams:

$$0.268 \text{ g Hg.} \times \frac{1000 \text{ mg}}{1 \text{ g}} = 268 \text{ mg Hg.}$$

(3) Select the temperature correction factor (that is, the weight of 1 microliter of mercury in milligrams) from below.

Temperature (degrees Centigrade)	Mercury (weight of 1 μl)* mg
20.0	13.55
21.0	13.55
22.0	13.54
23.0	13.54
24.0	13.54
25.0	13.54
26.0	13.53
27.0	13.53
28.0	13.53

*These values do not indicate the true densities and weights of water and mercury but include corrections for various factors, such as the coefficient of expansion of glass and the buoyant effect of air on the fluid weighed.

(4) The volume of the pipet is obtained by calculating the volume of mercury.

Example:

$$\text{Wt. of mercury in mg} \times \frac{1 \text{ microliter Hg.}}{\text{No. mg at T}^\circ} = \text{vol. Hg. in microliters.}$$

$$(\text{Wt of delivered Hg})\ 268 \text{ mg Hg} \times \frac{1\ \mu\text{l Hg}}{13.55 \text{ mg Hg}} = 19.8\ \mu\text{l vol}$$

The expected weight for 20 cubic microliters of mercury is 270.8 mg.

NOTE: For routine purposes, it is acceptable to use pipets within ± 1 percent of 20 microliters without making a correction. Therefore, pipets which deliver 268.1 to 273.5 mg of mercury would be acceptable. Discard unacceptable pipets.

2-7. Blood Cell Counting Chambers:

a. The most common type of hemacytometer consists of two counting chambers separated by grooves or canals. On the smooth glass surface of the counting chamber are straight lines etched into glass in a gridwork pattern. The Neubauer ruling, preferred for hematological work, consists of a gridwork with dimensions of 3 mm by 3 mm. It is further divided into 9 smaller squares with dimensions of 1 mm by 1 mm; 4 of these squares are used for the white count. The 8 outer squares are further subdivided into 16 squares 0.25 mm on a side. The central square is divided into 25 squares, 0.20 mm on a side, which are used for the red cell count. Thus the large squares are 1 square mm, the 16 small squares in the outer large squares are 1/16 square mm and the 25 central squares are 1/25 square mm (see figure 2-3).

b. The coverglass must be free of visible defects and must be optically plane on both sides within ± 0.002 mm according to the US Bureau of Standards. When the coverglass is placed on the platform the space between it and the ruled platform should be 0.1 mm.

2-8. Cleaning. Glassware can be cleaned in hot, soapy water and thoroughly rinsed in distilled water. Blood dilution pipets can be washed by flushing water and acetone through them. Ordinary household bleach can be used to remove blood clots in the bore of pipets. Dilution pipets are dry when the bead moves freely in the bulb. Glassware to be used for coagulation studies must be scrupulously clean. This glassware should be cleaned in nonorganic detergents and rinsed well with distilled water. The etched surface of the hemacytometer should be rinsed in water and blotted dry with lens paper to avoid marring or further etching of the lines on the surfaces. A method of cleaning small bore tubes and pipets (such as Wintrobe sedimentation rate tubes) is to attach a capillary pipet, by a rubber hose, to a water type suction pump. Attach the tube to the flat end of the pipet and hold

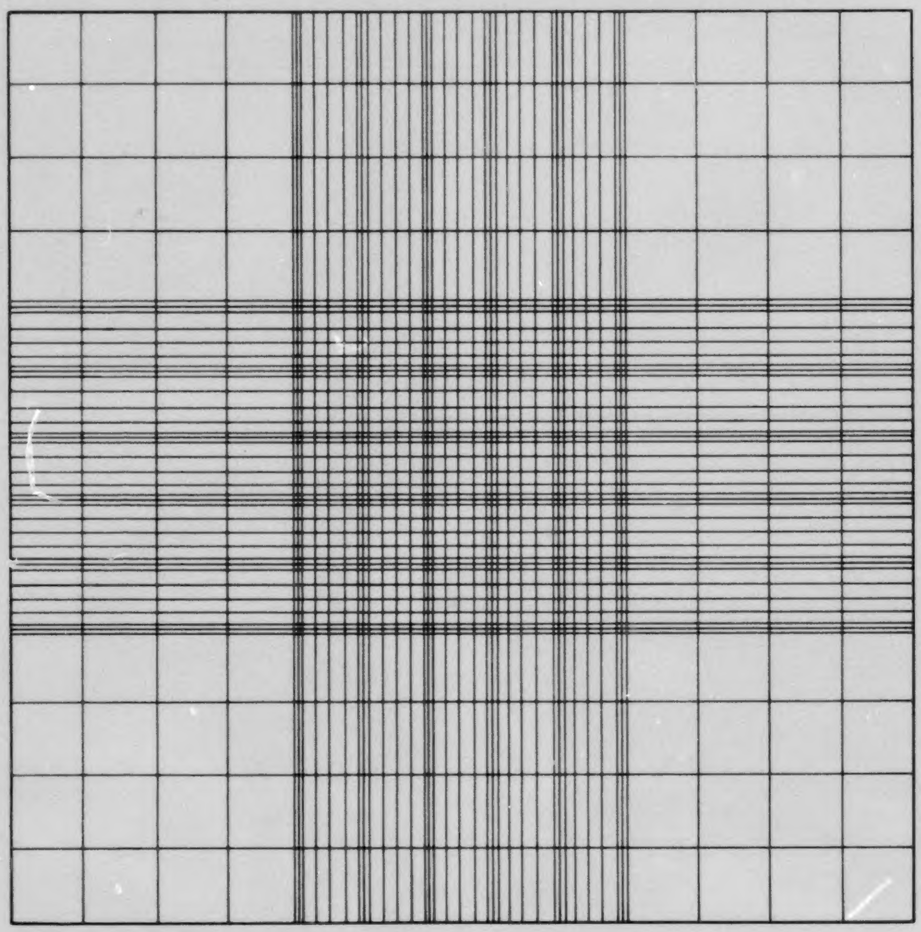


Figure 2-3. Rulings on a Hemacytometer.

the tube under water. Blood is drawn from the tube as water is drawn in. When the tube is clean, invert the tube, remove the residual water by suction, and allow to dry. Occasionally, the tube should be cleaned with dilute sodium hypochlorite (household bleach) to remove deposits of residual blood.

2-9. Preparation of Siliconized Glassware:

a. Coating Materials:

(1) Silicone (SC-87 Dri-Film available from General Electric Corporation, Silicone Products Division, Waterford, New York).

(2) Xylene.

(3) 10% Silicone Solution. Add 100 ml of concentrated silicone solution to 900 ml of xylene in a clean ground-glass-stoppered bottle. This solution can be used indefinitely.

b. Procedure:

(1) Handle the 10% silicone solution with rubber gloves and work in a hood equipped with an exhaust fan.

(2) Syringes, barrels, and plungers are coated with 10% silicone solution in a large beaker.

(3) To coat test tubes, fill one test tube with the 10% silicone solution. Rotate the tube and transfer to other test tubes.

(4) Pipets are coated by placing tip of the pipet about 2 inches into the solution. Draw the solution up with a suction bulb an inch below the mouth end.

(5) Allow the glassware to drain for 20 minutes after coating. Rinse well in tap water and then in three or four rinses of distilled water. Dry in an oven at 120° C.

c. Discussion:

(1) Glassware can be desiliconized by soaking in saturated sodium hydroxide for 10 minutes. Rinse in distilled water and dry in an oven.

(2) Silicone glassware can be used several times before recoating.

(3) All siliconized glassware should be prepared, washed, and stored in an area separate from regular glassware.

(4) As the silicone ages or a new batch is prepared, perform a clotting time on normal whole blood to check on the "wettability" of the coated glassware. In addition, water should have a flat or convex meniscus in a properly coated tube.

SECTION C—COMPOUND MICROSCOPE

2-10. Introduction. A modern microscope for use in the hematology laboratory is equipped with an illuminator system, a substage condenser system, an objective system, a projector (eyepiece or ocular system), an iris diaphragm, nicol prisms, a tubular barrel (monocular or binocular bodies), and a mechanical stage (see figure 2-4). A compound microscope uses a combination of lenses, the objective lens (lens closer to the object) and the ocular lens (lens closer to the eye) to project the image to the retina of the eye. The objective lens acts much like a small projection lens which projects an enlarged primary image near the top of the tubular barrel. This image, formed in air, is known as an "aerial image." This object is viewed through the projector or eyepiece which acts like a magnifier except that it magnifies an aerial object instead of an actual object. The final image projected on the retina of the eye is called a "virtual image" because the light rays appear to come from the image. The rays are actually created by an increase in magnification by the lens system.

2-11. Magnification:

a. Magnification in a microscope is limited to the useful magnification that can be achieved, that is, the ability to obtain fine detail of the object being examined. This ability to render visible the fine detail is the resolving power of the microscope. The resolving power of a microscope is dependent on the numerical aperture (N.A.) of the objective lens and condenser lens.

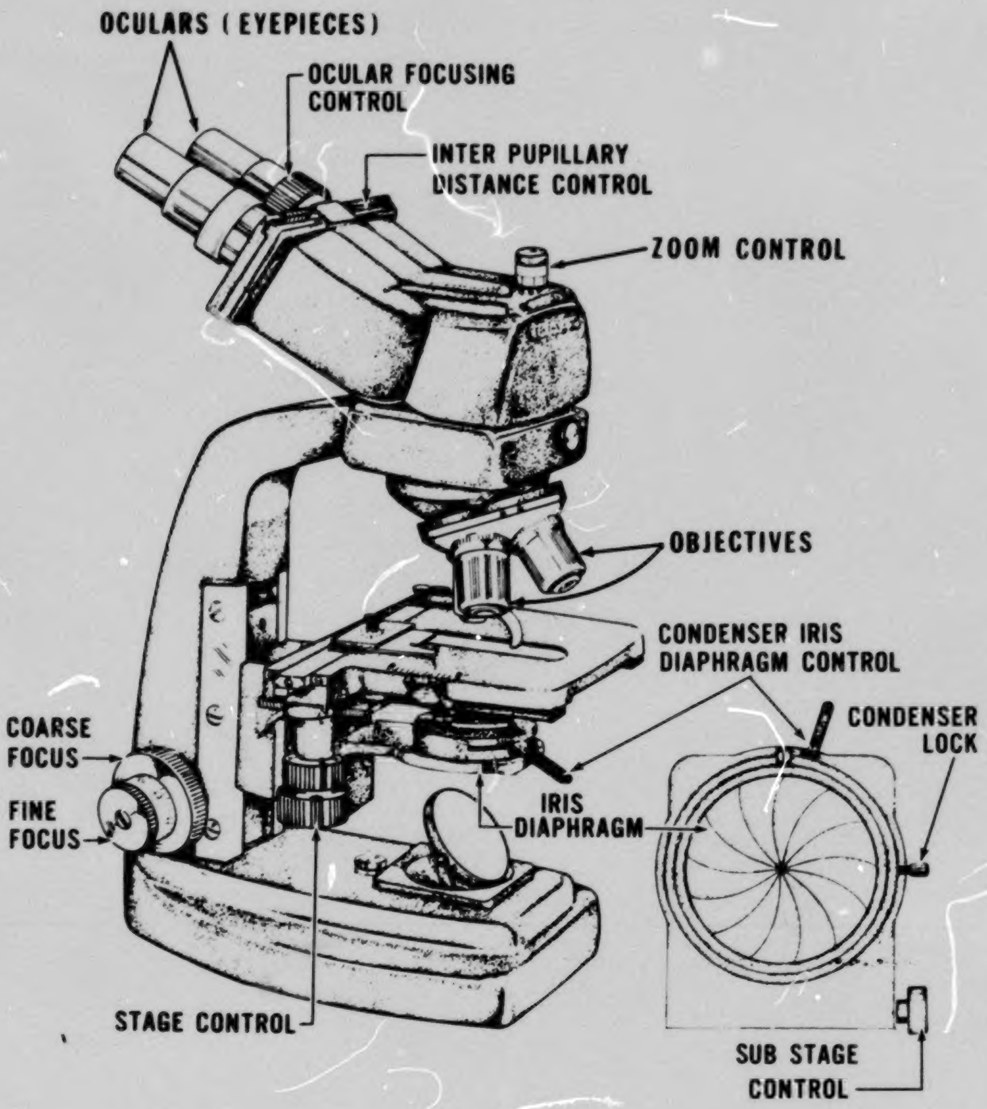

OCULARS (EYEPIECES)
OCULAR FOCUSING CONTROL
INTER PUPILLARY DISTANCE CONTROL
ZOOM CONTROL
OBJECTIVES
CONDENSER IRIS DIAPHRAGM CONTROL
CONDENSER LOCK
COARSE FOCUS
FINE FOCUS
IRIS DIAPHRAGM
STAGE CONTROL
SUB STAGE CONTROL

Figure 2-4. Compound Microscope.

Therefore, proper adjustment of these lenses is essential in order to obtain useful magnification.

b. Microscopes in general use in medical laboratories are provided with three objectives with focal lengths of 1.9 mm, 4 mm, and 16 mm, respectively. Microscopes are usually provided with 5X and 10X (most common) oculars. Multiplying the power of the ocular by the power of the objective gives the degree of magnification of the object under observation. The degree of magnification is expressed in diameters (refers to an increase in diameter). The ocular magnification, the millimeter length of the objective, its magnification power, and the total apparent increase obtained using oculars and objectives of the powers shown are given below:

Ocular	Objective	Magnification
10X	16 mm (10X)	100 diameters
10X	4 mm (43X)	430 diameters
10X	1.9 mm (97X)	970 diameters

Magnification is increased in practice by using a higher power objective. Most microscopes are equipped with a revolving nosepiece, and selection of an objective lens is done with ease.

2-12. Illumination:

a. Correct illumination of the object under study is an extremely important detail. Incorrect lighting of the object can lead to inaccurate results and conclusions. Correct illumination can be obtained from a concave mirror or substage light.

b. Illumination entering the microscope can be central or oblique. To obtain central illumination the light from the source must be reflected from the mirror directly into the microscope tube. Oblique lighting must be avoided because an object in the center of the field will sway from side to side when the fine adjustment is rotated. Oblique light is usually no problem with substage illuminators.

c. Regulation of the amount of light admitted is accomplished by the iris diaphragm in the substage condenser. The size of the opening in the diaphragm is controlled by a lever on the side of the condenser. The lever of the iris diaphragm should never be forced to the full limit in either direction. Doing so may damage the delicate leaves of the diaphragm. Generally, when observing liquid preparations under low power, the diaphragm opening should be partially closed. Under the high dry objective, the diaphragm is generally opened to a greater degree to allow more light to pass through the material. When observing stained preparations under the oil immersion objective, the iris diaphragm is usually opened wide.

d. The substage condenser functions to direct a light beam of the desired numerical aperture (N.A.) and field size onto the specimen. The size of the opening in the condenser together with its position up or down controls the light entering the system. When the condenser is close to the stage, concentration of light is greater; as the condenser is moved downward, less light passes upward through the object under observation.

e. Improper illumination is indicated when: (1) dark points or shadows appear in the field; (2) the outline of an object is bright on one side and dark on the other; or (3) the object appears to be in a glare of light. This can usually be corrected by changing the position of the mirror, by reducing the amount of light by adjusting the size of the opening in the iris diaphragm, or by raising or lowering the condenser.

2-13. Focusing. Focusing can be defined as the adjustment of the relationship between the optical system and the object so that a clear image is obtained. Several important rules to be observed when focusing the microscope on the preparation are:

a. After the object is mounted on the stage, the objective to be used is turned into line with the eyepiece.

b. Movement of the objective is accomplished by revolving the nosepiece. The nosepiece is provided in order to enable rapid, convenient substitution of one objective for another. This change is effected by grasping two of the objectives between the thumb and forefinger of the right hand and rotating them until the desired objective is brought into line with the axis of the body tube. It is very important that exact alinement be obtained. The correct setting is indicated by a slight "click" as the objective comes into position.

c. Whenever the nosepiece is revolved, its movements should be observed to make certain that the objectives do not come into contact with the object. Some microscopes are not parfocal; that is, objects in focus under low power will not be in focus when the nosepiece is rotated to a higher power of magnification. It may, therefore, be necessary to refocus when changing to higher magnification. In microscopes which are parfocal, it is possible to swing other objectives into place without touching the coarse adjustment and with only a slight turn of the fine adjustment knob required to restore perfect focusing.

d. To bring an object into focus, watch from the side and use the coarse adjustment to lower the objective until it is below the point at which the object would normally be expected to come into view. NOTE: To avoid damage to slide or microscope, view from side for preliminary focusing. Then, using the coarse adjustment and at the same time looking through the ocular, raise the objective very slowly until the field comes into view. Further adjust to the best image, using only the fine adjustment.

e. In focusing upward with the fine adjustment, the object will first appear in faint outline, then gradually more distinctly, and finally, sharply defined. If the adjustment goes beyond the point of sharp definition, return to the point of greatest clarity by using the fine adjustment.

f. Never move an objective downward while looking through the eyepiece. When the objective is moved downward, always observe the downward motion with the eye held level with the microscope stage. Failure to observe these precautions can result in damage to the lens of the objective or the object under study.

2-14. Care of the Microscope. The microscope is an instrument of precision with many delicate parts, and it must be handled with the utmost care. Care should not be confined to the optical elements alone. The microscope is a combination of optical and mechanical excellence, one complementing the other. The following precautions should always be observed in the care of the microscope:

a. No unauthorized person should manipulate the microscope.

b. Keep the microscope as free from dirt and dust as possible. Dusty lenses produce foggy images, while dust in the focusing mechanisms causes excessive wear of those parts.

c. The microscope should always be covered when not in use.

d. Care should be taken to prevent all parts of the microscope from coming into contact with acid, alkali, chloroform, alcohol, or other substances which corrode metal or dissolve the cementing substance by means of which the lenses are secured into the objectives and oculars.

e. Always carry the microscope with two hands by the arm and base.

f. Avoid sudden jars, such as placing the microscope on the table with undue force.

g. No dust should be permitted to settle on the lenses nor should the finger come in contact with any of the surfaces.

h. The lens system should never be separated, as the lenses are liable to become decentered and dust can enter.

i. Avoid all violent contact of the objective lens and the coverglass.

j. Keep eyepieces in the microscope at all times to keep free of dust.

k. To remove dust, brush the lenses with a camel's hair brush. Avoid hard wiping, as dust is often hard and abrasive.

l. Xylol is the only agent which should be used in cleaning lenses or removing oil from objectives. Only small amounts of xylol are necessary and should be used with care.

m. When sewing machine oil is used to lubricate moving parts of the microscope, all excess should be wiped off to prevent the collection of grit and dust.

n. The microscope should be protected against direct sunlight and moisture.

o. In very warm, humid climates, microscopes should be stored in dry cabinets when not in use. Such cabinets should be reasonably airtight, equipped with a light bulb to supply heat, and several cloth bags containing a hygroscopic salt such as calcium chloride to absorb moisture. In warm, humid climates, the lenses of unprotected microscopes can be attacked by certain fungi which etch glass and ruin the lenses.

p. After use, always turn the nosepiece to a position which brings the low power objective into direct line with the opening in the substage condenser. If this precaution is not taken, the longer higher powered objectives can accidentally come into contact with the condenser lens.

q. The entire microscope should be cleaned frequently to remove dust, finger marks, oil, grease, and remnants of specimens. All parts of the microscope should be kept scrupulously clean at all times.

r. Never tamper with any of the parts of the microscope. If the instrument does not seem to be functioning properly, immediately call the matter to the attention of the laboratory officer.

s. Maintenance of the microscope should be done in accordance with the manufacturer's booklet of instruction.

t. Immediately after use, the oil immersion objective must be wiped clean of oil with a soft, absorbent lens paper.

2-15. Types:

a. Binocular. This type of microscope is preferred since it gives a more natural and restful condition of observation. A beam-dividing prism and three mirrors divide the light equally, sending half to the left eye and half to the right eye. The coating on the mirrors is enhanced by aluminum to increase the reflectivity. The binocular body is protected by coverglass seals to keep dust from entering. The binocular compound microscope is the preferred microscope for routine hematology.

b. Phase. Phase microscopy is becoming increasingly prevalent in platelet counting. In bright-field illumination, a completely transparent specimen is difficult to see in any detail. By using phase contrast, transparent living objects can be studied. Phase microscopy operates on the principle that if a portion of light is treated differently from the rest, and caused to interfere with the rest, it produces a visible image of an otherwise invisible transparent specimen. Phase contrast accessories are available from the standard optical companies.

SECTION D—CENTRIFUGES

2-16. Types:

a. Table Top Models. These units are mounted on rubber feet which absorb vibration. The speed is controlled by means of a rheostat on the front panel. Top speeds of centrifuges will vary and the top speed of a particular instrument should be known in order to use the speed control device. Those centrifuges have adapters to hold 6 tubes and adapters for 12 tubes.

b. Floor-Mounted Models. The heavier floor-mounted models accommodate a large number of tubes at one time. The top speed of these instruments is higher than that of table models. Because of their increased inertia, they are equipped with a brake to facilitate stopping. In these units, the tubes are placed in balanced receptacles which are mounted on spokes emanating from a central hub.

c. Microhematocrit Centrifuge. This centrifuge is a special type of high speed centrifuge employed to spin capillary tubes (see figure 2-5). The circular tube holder on this centrifuge is flat, surrounded by a rubber ring. It has a capacity of 24 capillary tubes. After a capillary tube is filled with blood, it is closed with a commercial plastic sealing material. During centrifugation the sealed end is always placed in position facing toward the outside of the holder plate. Most centrifuges of this type spin the tubes at 10,000 rpm.

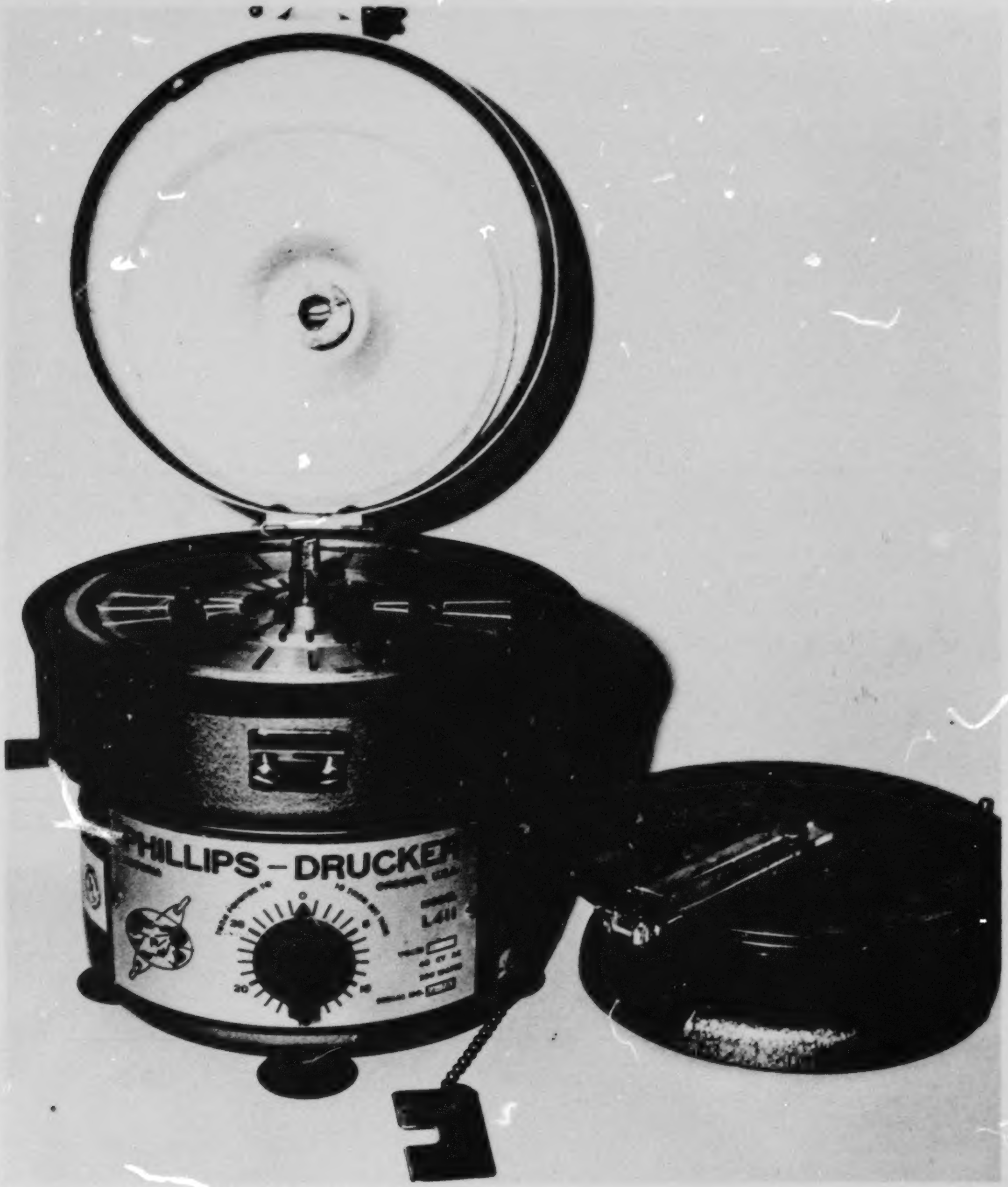


Figure 2-5. Microhematocrit Centrifuge and Microhematocrit Reader.

2-17. Precautions. In all instances where centrifugation is required, careful attention must be given to balancing the units. This means that tubes must be placed exactly opposite each other, they must be of identical weight, and they must contain the same amount of fluid. If at all possible, centrifuges should be equipped with tachometers so that speed may be checked and controlled. Certain procedures, such as hematocrits, require a critical relative centrifugal force (RCF or G). To determine the RCF (or G) for these procedures, consult the serology manual or a nomograph. The inside of the centrifuges should be occasionally cleaned to prevent dust particles from being blown into specimens. The lid on the centrifuge should be closed and locked before and during operation. Only open the lid when the centrifuge has stopped rotating.

SECTION E—AUTOMATED EQUIPMENT

2-18. Introduction. In recent years instrumentation has been developed to automate many hematological analyses. The use of automated equipment in the laboratory has increased the number of analyses performed by decreasing the man-hours spent on the analyses. These instruments perform various functions such as: dilutions, staining, cell counts, hematocrit, hemoglobin, coagulation studies, and indices. The type of instrument used by a particular laboratory depends on the size of the daily workload, the complexity of the method used, and the technical ability of the technician performing the analysis. If the number of hematology requests received is 30–40 a day, it would be advantageous to have automatic diluters, pipetters, and electronic cell counters for blood cell counts and hemoglobin determinations. When the number of routine requests approaches 100–150 per day, an automatic hematology system should be considered.

2-19. Dilutors. Automatic dilutors assist in the processing of large numbers of specimens by reducing technician time per test. Repetitive dilutions can be accomplished rapidly with an instrument similar to the one illustrated in figure 2-6. The concept of an automatic dilutor involves the sampling from a larger volume and the dilution of the sample. The automatic dilutor is essentially two syringes that can be operated in a systematic manner. One syringe measures and draws the sample, the other syringe measures the diluent. Both syringes can be set to draw a metered amount repeatedly within an allowable range of error. A daily calibration check of this instrument is recommended to insure accuracy.

2-20. Slide Stainer. The automatic slide stainer is a coordinated three-phase operation that fixes, stains, buffers, rinses, and dries differential slides at a rate of approximately one per minute. Stain packs are available with this instrument, but satisfactory Wright stain and buffer can be prepared in the laboratory. The slide stainer is illustrated in figure 2-7.

2-21. Counting Devices:

a. Introduction. Two automated methods are available for counting blood cells. The optical system is based upon the production of light impulses. In this system, the cells are diluted and drawn through the counting zone by a positive displacement metering pump. As cells pass through the counting area, they produce photoelectronic impulses which can be counted. Another method of automating blood cell counts utilizes the principle of resistance in an electrical field. Since blood cells are poor electrical conductors, they act as resistors to current flow. As more cells pass into the electrical field they offer correspondingly more resistance. The change in the current flow caused by the change in resistance is sensed and counted by a digital counting apparatus. The digital counter is designed to produce numbers in a range which approximates the number of blood cells that cause the resistance.

b. Coulter Counter (Model FN):

(1) The Coulter Counter (see figure 2-8) operates on the principle of resistance. A suspension of blood cells in an electrolyte (that is, saline) is drawn through an aperture with electrodes on each side to form an electrical circuit. As the blood cells pass through the aperture, the mass of the cell changes the resistance between the electrodes. The change of resistance alters the

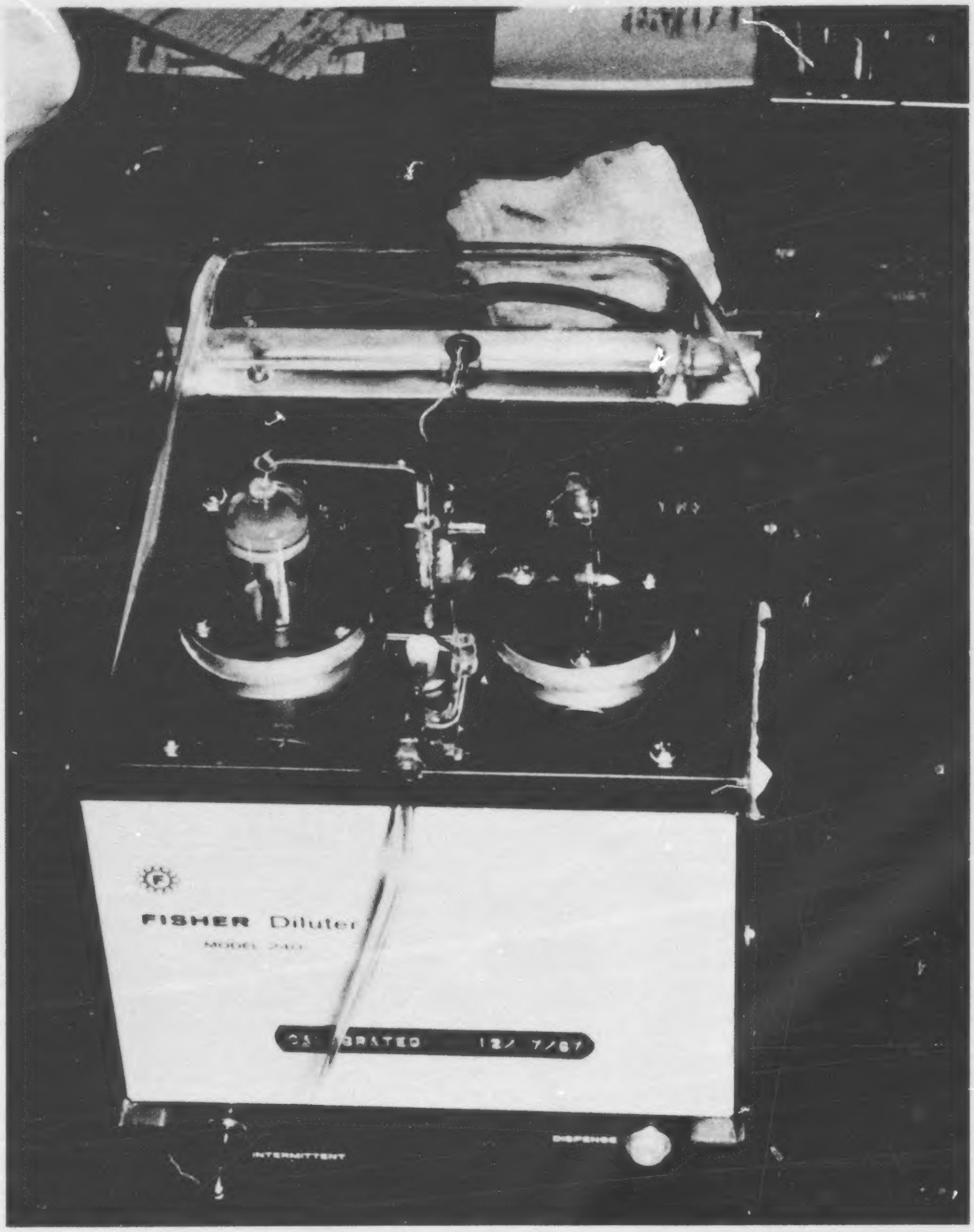

FISHER Diluter
CALIBRATED 12/ 7/67
INTERMITTENT
DISPENSE

Figure 2–6. Automatic Dilutor.

current flow and causes electronic pulses as a result of the variation in the field. The changes are amplified, inspected, and counted electronically. The end product is a number which represents the number of blood cells in the sample being counted. By arranging the pulses, the sampling volume, and the sampling time, the Coulter Counter reports directly the number of cells per cubic millimeter. A schematic of the operation of the

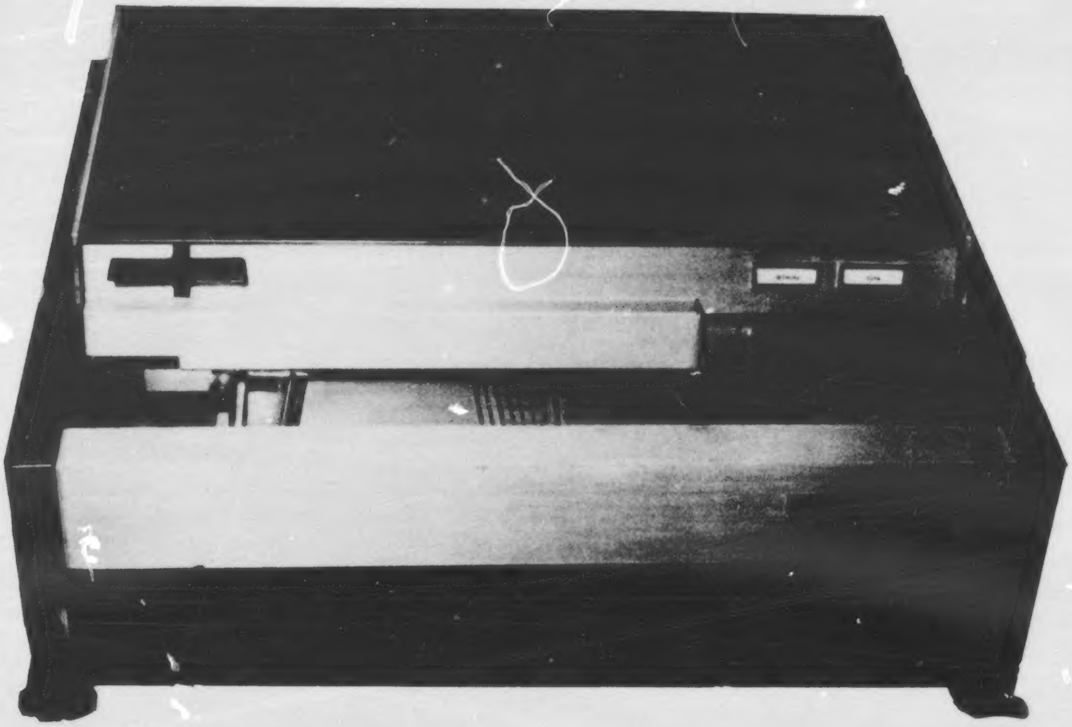


Figure 2-7. Automatic Slide Stainer.

Coulter Counter is illustrated in figure 2-9. When the valve is opened, the mercury falls, thus creating a vacuum in the sampler. This change in pressure causes cells in the sample to be sucked through the aperture and into the sampler. The removal of cells from the sample causes a change in electrical conductivity between the two electrodes. This change is amplified and displayed on the scope. It is further amplified and registered on the digital counter. The higher the count, the greater the probability that more than one cell will enter the aperture at one time (coincidence passage). For this reason, WBCs over 10,000 and all RBCs are corrected for coincidence passage.

(2) Adequate maintenance of the Coulter Counter will reduce incidence of instrument failure. The following should be done on a daily basis:

(a) Observe the mercury traveltime in the manometer. If the mercury column does not move, moves erratically, or flows quickly into the aperture tube, the manometer needs cleaning.

(b) Record a background count with diluent and diluent plus saponin at the WBC threshold setting.

(c) Record the control suspension counted at the RBC and WBC setting.

(d) Flush orifice with dilute sodium hypochlorite (bleach). Flush the system thoroughly with distilled water and then saline.

(3) In addition to the daily maintenance, the following maintenance should be performed once a week:

(a) Oil the vacuum pump.

(b) Clean the orifice; be very careful with the orifice insert.

(c) Check threshold zero.

(4) Check the threshold plateau every month.

(5) Once every 6 months perform the following maintenance:

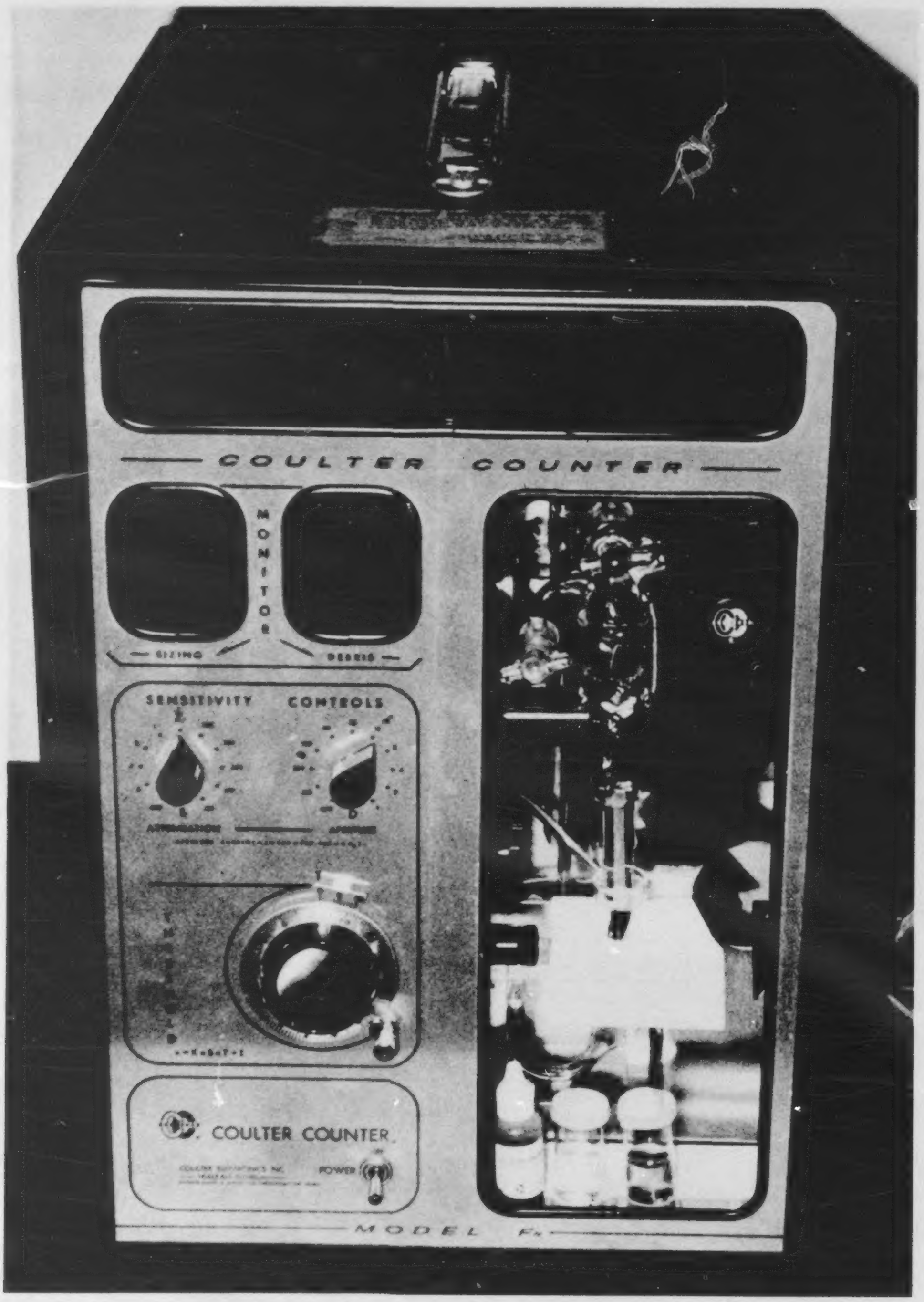

COULTER COUNTER
MONITOR
SIZING
DEBRIS
SENSITIVITY
CONTROLS
COULTER COUNTER
POWER
MODEL FN

Figure 2-8. Coulter Counter Model FN.

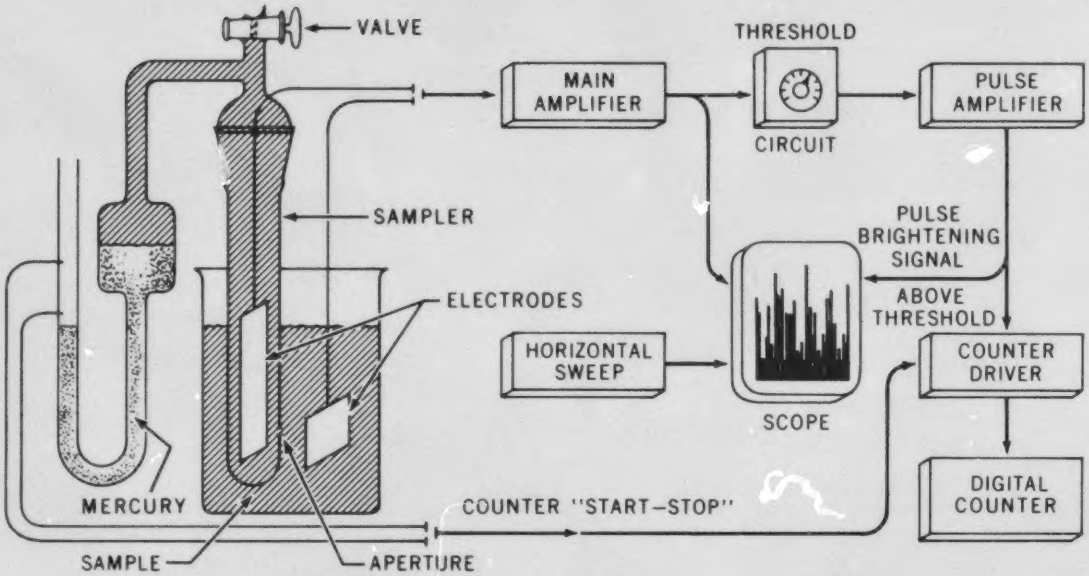

VALVE
THRESHOLD
MAIN AMPLIFIER
CIRCUIT
PULSE AMPLIFIER
SAMPLER
PULSE BRIGHTENING SIGNAL
ELECTRODES
ABOVE THRESHOLD
HORIZONTAL SWEEP
COUNTER DRIVER
SCOPE
DIGITAL COUNTER
MERCURY
COUNTER "START-STOP"
SAMPLE
APERTURE

Figure 2-9. Schematic Diagram of the Coulter Counter.

(a) Change the latex tubing.

(b) Clean the manometer.

(c) Check the calibration factor for the instrument.

c. Fisher Autocytometer. This instrument is a photoelectronic counting device which uses an optical sensing system and an electronic counting system. A schematic presentation of this arrangement appears in figure 2-10. The blood cells are diluted, syringe fed into the counting area, and inspected by a reversed dark field microscope arrangement. When no particles are present in the sample, the narrow light beam from the lamp passes straight through to a dark-field disc, which blocks further passage. However, any suspended particles, such as blood cells, will interrupt the light beam. Scattering of the light beam due to the mass of each blood cell causes a light flash to pass around the dark-field disc. The light flash is focused into a detection system and converted into an electrical pulse that can be counted.

d. Problems of Electronic Counters:

(1) Dilution inaccuracies often result in erroneous results.

(2) False impulses resulting from electronic problems such as interference from other machines can cause erroneous results.

(3) Contaminating particles in apparently clear diluent are another source of impulse. Background counts must be made daily on all diluents.

(4) White blood cell counts are not accurate over 10,000 per cu mm and must be diluted with saline.

(5) High platelet counts or nucleated erythrocytes can cause erroneously elevated white blood cell counts. A correction must be made if these cells are observed on the blood film.

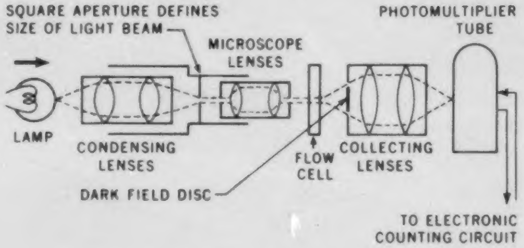

SQUARE APERTURE DEFINES SIZE OF LIGHT BEAM
MICROSCOPE LENSES
PHOTOMULTIPLIER TUBE
LAMP
CONDENSING LENSES
FLOW CELL
COLLECTING LENSES
DARK FIELD DISC
TO ELECTRONIC COUNTING CIRCUIT

Figure 2-10. Schematic Diagram of a Photoelectric Particle Counter.

(6) Standardization is required with hemacytometer results.

(7) Bubbles must be avoided since they will be counted as cells.

(8) The orifice of the counter can become plugged. All equipment used with the counter should be cleaned.

(9) The equipment has a high initial cost; meticulous care is required for the proper maintenance of the counter.

2-22. IL Hemoglobinometer:

a. This instrument utilizes a direct digital presentation for a rapid readout of hemoglobin concentration. It contains a high-precision, interference filter with a wavelength of 548.5 nm. In principle, this instrument measures the three naturally occurring hemoglobin pigments (oxyhemoglobin, reduced hemoglobin, and carboxyhemoglobin). This instrument is not suitable for rapid measurement of cyanmethemoglobin and hence cannot be effectively used for certain artificial standards or survey samples which contain variable quantities of methemoglobin.

b. Operation of the machine is simple. Prior to actually testing a specimen, the machine must be set using a baseline saline solution. This is done by running saline through the machine and adjusting to zero with the zero control. Next, the machine is calibrated using an amaranth dye solution. The machine is adjusted to 15 grams per dl with this calibration solution. The machine is now ready to accept the actual specimen. An internal pump draws the specimen up through the long slender sampler on the front of the machine. An aliquot of a hemolyzing solution is then pumped from the reservoir. The specimen is mixed, hemolyzed, and passed through a filtered cuvet for reading. The reading goes to an analog computer which converts it into a numerical reading that is displayed on the digital readout register.

2-23. Fibrometer. The Fibrometer (see figure 2-11) is an automatic instrument used to perform routine coagulation tests. This instrument consists of a precision coagulation timer, a 37.2 ± 0.7°C incubator block, and an automatic pipet. The automatic pipet, attached to the Fibrometer by an electric cord, dispenses reagents or plasma and automatically activates the Fibrometer. A plastic cup beneath the probe on the Fibrometer is charged with reagents. When the plasma is delivered with the switch on the pipet in the "ON" position, the timer is started and the electrodes drop into the mixture. The first fibrin formation on the electrodes closes the circuit and stops the timer. The elapsed time in seconds is read directly from the digital readout register on the timing device. This instrument is manufactured by the Baltimore Biological Laboratory.

2-24. Hematological Systems. Complete hematological systems have been developed that perform seven hematology parameters—red blood cells count, white blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration. These instruments automatically dilute and analyze the specimen, and tabulate the results on data forms.

a. The Technicon SMA 7A. This system is a sequential multiple autoanalyzer consisting of: a sampler containing 40 cups, a proportionating pump which precisely propels reagents and samples, a manifold consisting of constant diameter plastic tubing and mixing coils, an optical-electronic cell counter, an electrical conductance flow cell to determine hematocrit, a colorimeter for hemoglobin determination, and recorder to measure the data. The sample is split into four streams to perform (1) RBC count, (2) WBC count, (3) hematocrit, and (4) hemoglobin. The instrument performs four separate determinations at a rate of 60 samples per hour or 240 tests per hour.

b. Model S Coulter Counter. This instrument (see figure 2-12) utilizes automatic

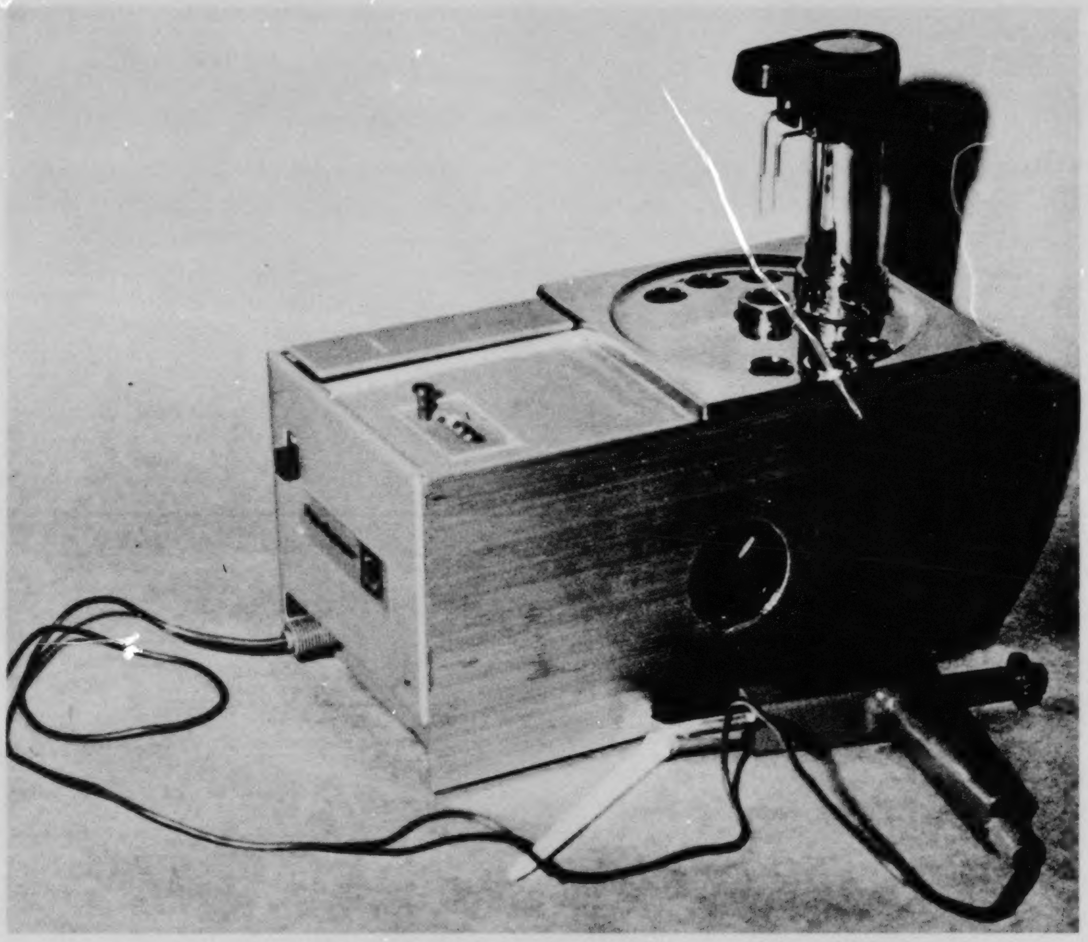


Figure 2-11. Fibrometer.

dilution analysis to perform the seven hematology parameters. The RBC and WBC counts are performed in triplicate. If there is any disagreement due to debris or other error, the technician will be alerted by an alarm system. The patient sample is introduced to the sample aspirator and the patient identification card is submitted to an automatic printer. The instrument is activated, it aspirates a sample, it performs the necessary dilutions, and the results are printed automatically on the patient's identification card. The Coulter Model S analyzes and records the data quickly and accurately.

2-25. Maintenance of Automated Hematological Equipment. Operation of all laboratory equipment according to the manufacturers' instructions is imperative for valid results. In addition, periodic maintenance and cleaning following manufacturers' and internal operating procedures must be performed to keep the equipment in operating condition. All operation and maintenance must be performed by competent personnel.

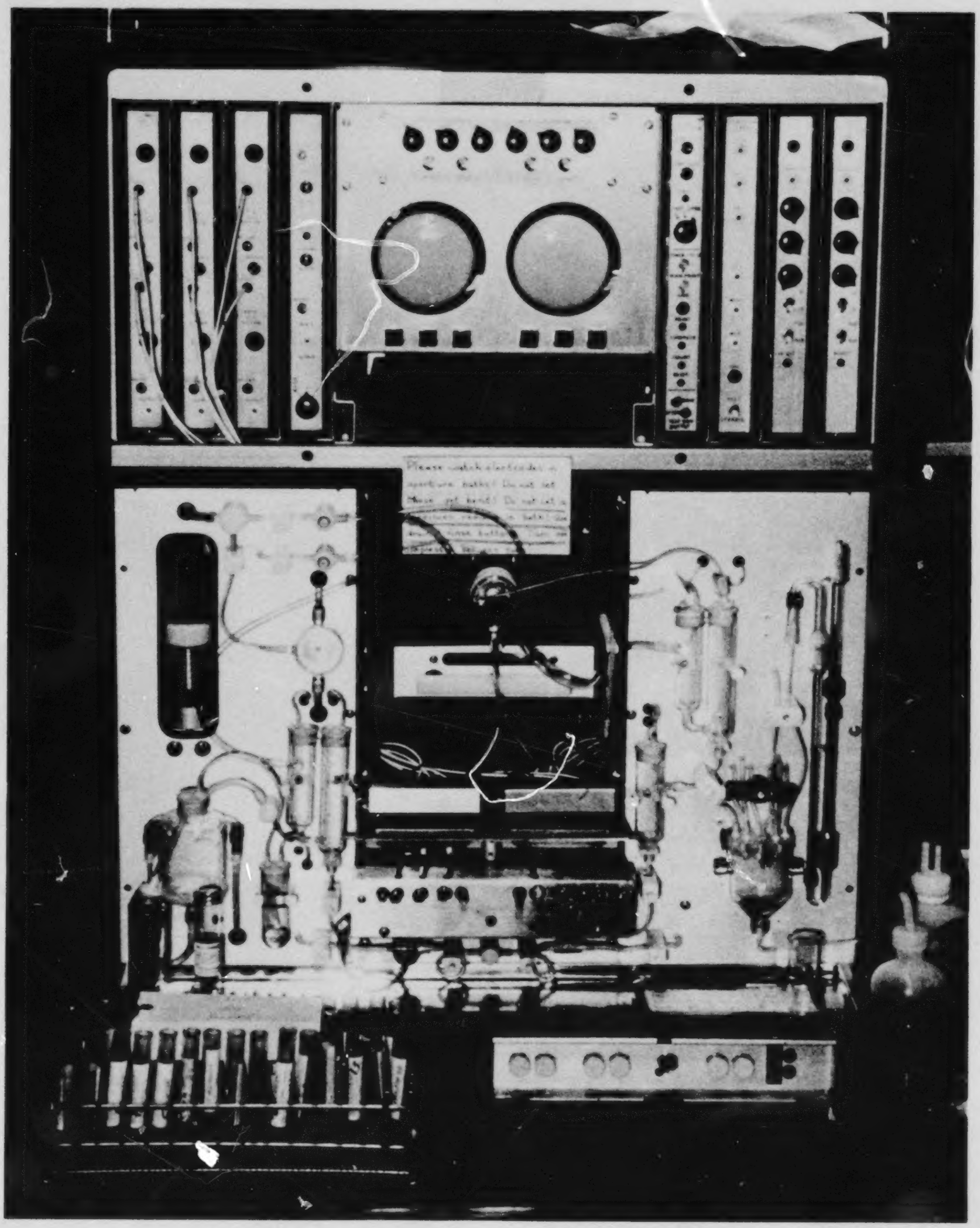


Figure 2-12. Coulter Model S.

Chapter 3

BASIC HEMATOLOGICAL PROCEDURES

SECTION A—COLLECTION OF BLOOD SPECIMENS

3–1. Introduction:

a. Hematological laboratory procedures are based upon the examination of blood specimens. To obtain valid test results, specimens must be properly collected, processed, and recorded. Blood specimens are usually obtained by either venous or capillary puncture. The source of the specimen is determined chiefly by the quantity of blood required to perform the laboratory procedures and the age and condition of the patient.

b. There is generally little difference in blood counts performed on venous or capillary blood if a free-flowing capillary blood specimen is obtained. Valid blood counts cannot be made when capillary specimens are not taken from a free-flowing sample or when they are obtained from cyanotic or calloused areas or areas of local stasis. White blood cell counts made on blood obtained from such sources can vary as much as 1000—1500 cells per cu mm from their real value. For general purposes, however, venous samples are preferable since they allow for multiple and repeated hematological examinations and provide a sufficient quantity of blood for performing any other required laboratory procedure. Further, with venous blood the chances of error are reduced because operations are made under ideal conditions and repeat operations are possible. In situations where there are limitations on the quantity of blood which can be obtained, that is, in small infants or extensive burn cases, microquantitative methods are satisfactory for performing an analysis on a specimen obtained by capillary puncture.

3–2. Venipuncture:

a. Site. To obtain blood by venipuncture, draw the specimen directly from a patient's vein with a sterile hypodermic needle and syringe or a vacuum blood sample device. In adults use the veins located in the proximal forearm or antecubital space as illustrated in figure 3–1. In infants employ the jugular of femoral vein for the venipuncture. The vein selected should be large, readily accessible, and sufficiently close to the surface to be seen and palpated. If venipuncture poses a problem due to the age of the patient, sclerotization due to repeated venipuncture, or any other unusual circumstance, the technician should consult a physician concerning the procedure. UNDER NO CIRCUMSTANCES SHOULD A TECHNICIAN WITHDRAW BLOOD FROM A SAGITTAL SINUS, JUGULAR VEIN, OR FEMORAL VEIN. This should be left to

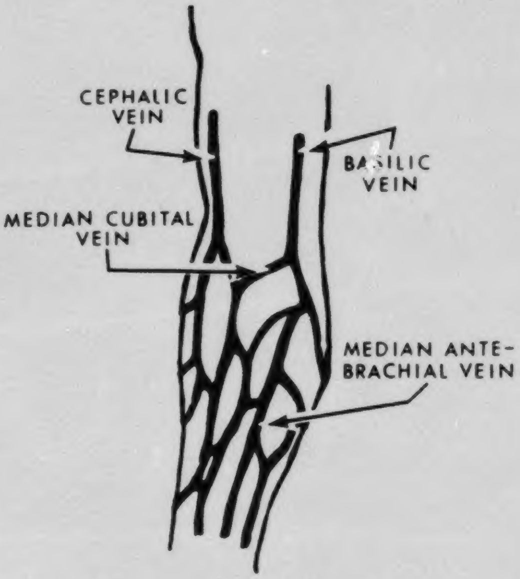


Figure 3–1. Site of Venipuncture.

the discretion of the physician in charge. Occasionally, the best vein is found on the hand, leg, or foot. These areas are more sensitive, and the veins are not as firmly anchored as those of the arm. Veins can become distended and easier to enter by allowing the arm to hang down for 2 or 3 minutes, by massaging the blood vessel toward the body, or by gently slapping the site of puncture. Young and vigorous persons usually have elastic veins well filled with blood. Elderly or debilitated persons can have sclerosed or fragile veins, which are hard to enter or which collapse easily.

b. Equipment. All syringes, needles, lancets, and other instruments used for the collection of blood specimens must be sterile. Disposable syringes or blood collection sets with vacuum tubes are available through normal supply channels. These should be used whenever possible. Aseptic technique is necessary to prevent the possible transmission of homologous serum hepatitis. The following equipment is necessary to perform a venipuncture:

(1) Isopropyl alcohol, 70%. Dilute 726 ml of 95% isopropyl alcohol to 1 liter with distilled water.

(2) Tourniquet.

(3) Alcohol sponges.

(4) Sterile syringes or vacuum blood sample devices.

(5) Gauze pads, 2 x 2 inches.

(6) Needle, 1 to 1 1/2 inches long, 19–23 gauge.

(7) Suitable blood collection tubes and labels.

c. Preparation:

(1) Cleanse hands thoroughly with soap and water.

(2) Place an identifying label on the blood collecting tube.

(3) Assemble the sterile needle and syringe. If a vacuum system is used, screw the needle into the plastic holder. Always leave the vial covering over the needle when not in use.

(4) Inspect the needle bevel and point against a light background. The bevel should be smooth and shiny and the point sharply tapered. Discard damaged needles.

(5) Check to be sure that the syringe works smoothly and that the needle is not plugged or burred. The syringe and needle must be dry to avoid hemolysis of the red cells. The plunger must match the syringe and must be pushed firmly to the bottom of the cylinder to prevent injection of air into the vein.

d. Syringe Procedure:

(1) Place a tourniquet around the patient's arm above the elbow tightly enough to check venous circulation, but not so tightly as to stop arterial flow. CAUTION: Do not allow the tourniquet to remain in place for more than 2 minutes. Check the pulse at the wrist to make sure that arterial circulation is not cut off.

(2) Instruct the patient to open and close the fist several times to increase circulation.

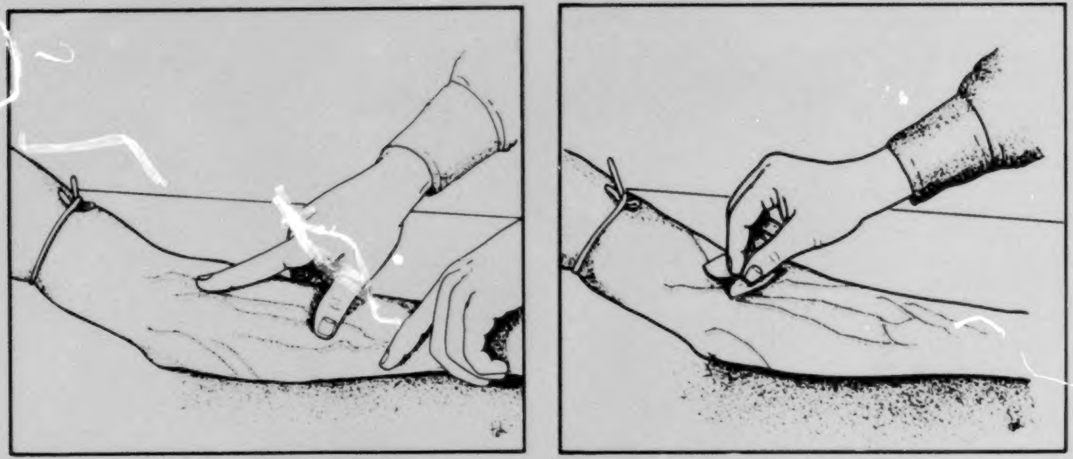
(3) By inspection and palpation locate the desired vein, determine the direction of its course, and estimate its size and depth (see figure 3–2a).

(4) Cleanse the skin over the selected vein with gauze soaked in 70 percent isopropyl alcohol. Wipe off excess alcohol with a sterile dry gauze. Do not contaminate the area after cleaning (see figure 3–2b).

(5) Have the patient "make a fist" and straighten the arm. Frequently it is helpful to have the patient place the clenched fist of the other hand under the elbow to straighten the arm.

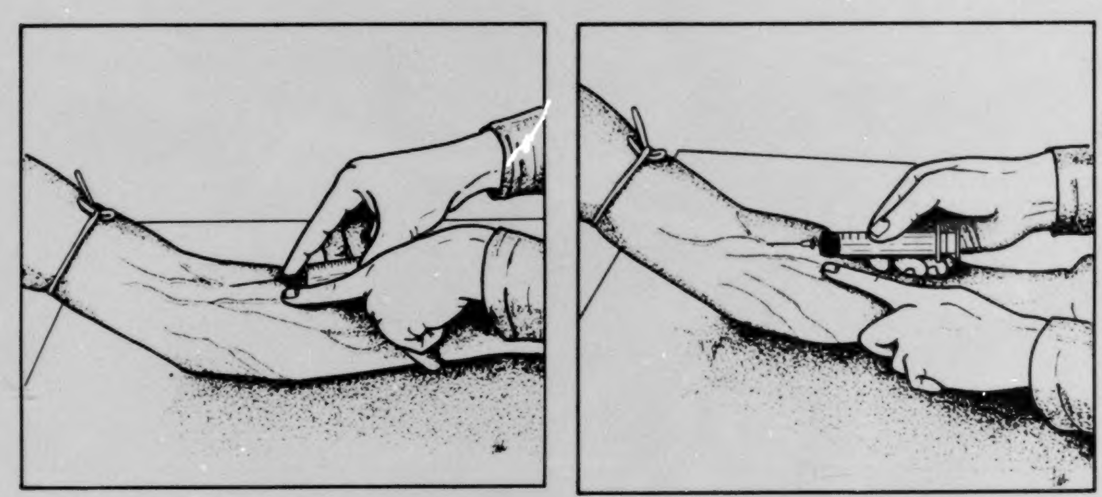
(6) Grasp the syringe in the right hand and place forefinger on the hub of the needle to guide it. Grasp the forearm with the left hand about 2 inches below the area to be punctured and hold the skin taut with the thumb (see figure 3–2c).

(7) With the needle bevel up, parallel



a. Locate the vein

b. Clean the puncture site



c. Guide needle toward the vein

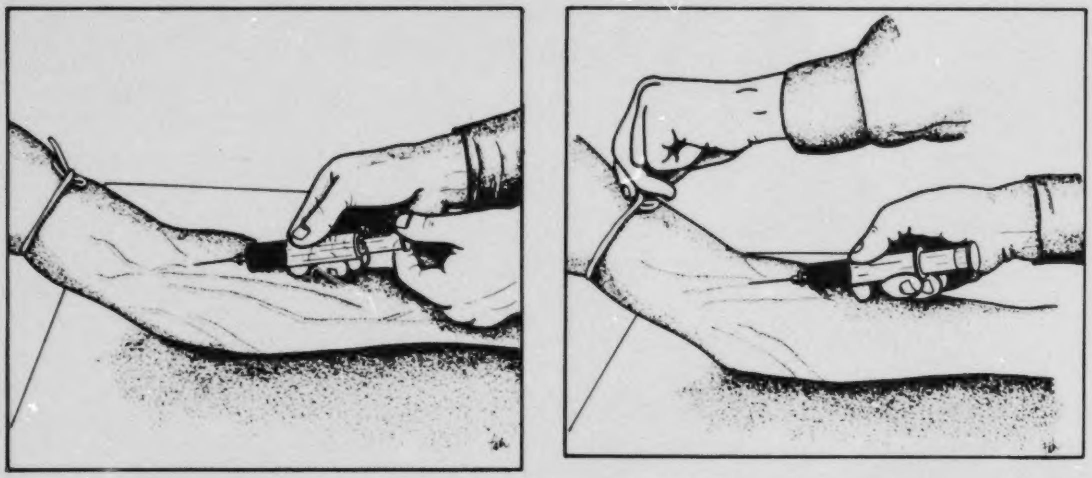
d. Insert needle into the vein

Figure 3-2. Venipuncture Procedure.

to, and alongside the vein, insert the needle quickly under the skin and then into the vein. The insertion into the skin and vein can be performed in one complete motion (see figure 3-2d). After entry into the vein, blood will appear in the needle hub. Do not probe or move the needle horizontally, as discomfort and possible nerve damage may result.

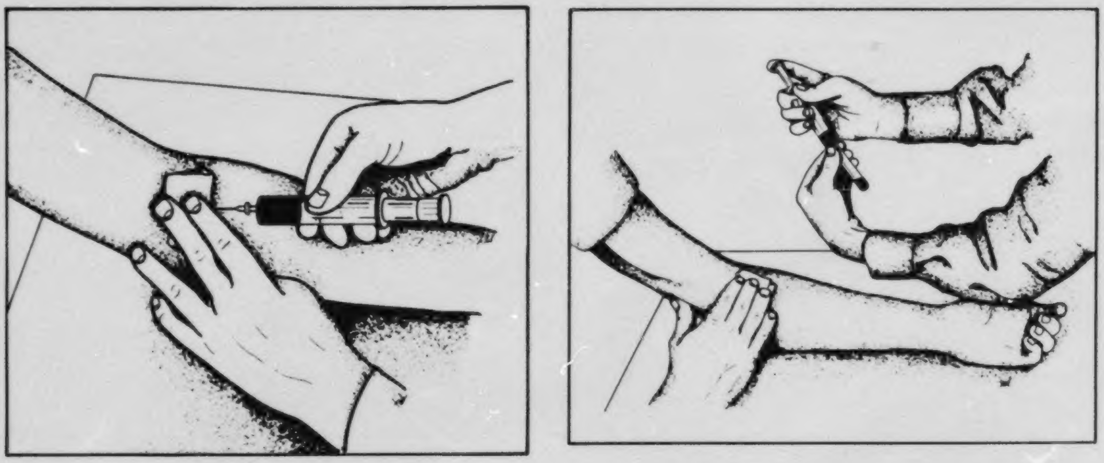
(8) Aspiration of the blood is accomplished by gently pulling upon the syringe plunger (see figure 3-2e). The syringe barrel should be held steady during this process. Withdraw the desired quantity.

(9) Remove the tourniquet (see figure 3-2f). This must be done prior to withdrawing the needle from the vein.



e. Aspirate the blood

f. Remove the tourniquet



g. Place a sterile gauze pad over the site and withdraw the needle

h. Have the patient extend the arm and maintain light pressure on the site

Figure 3-2. Continued.

(10) Place a sterile gauze pad over the point where the needle enters the skin and deftly withdraw the needle simultaneously putting pressure on the site (see figure 3-2g).

(11) Have the patient extend the arm and maintain light pressure on the gauze pad over venipuncture site (see figure 3-2h).

e. Vacutainer Procedure:

(1) Place the Vacutainer tube in the holder until the rubber stopper reaches the

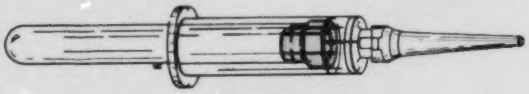


Figure 3-3. Vacutainer System.

guideline. The short needle should be embedded in the stopper, but the needle must not break the vacuum (see figure 3-3).

(2) Follow steps 1-6 in paragraph 3-2d.

(3) Enter the vein with the needle parallel to and alongside the vein. Probing or horizontal movement of the needle while under the skin must be avoided. Release the tourniquet.

(4) After entry into the vein push the tube all the way into the holder; vacuum is broken, and blood flows freely into the tube.

(5) Other tubes can be inserted in the same manner if needed.

(6) Place a sterile gauze pad over the point where the needle enters the skin and deftly withdraw the needle, placing pressure on the site.

(7) Have the patient extend the arm and maintain light pressure on the gauze pad over the venipuncture site.

f. Discussion:

(1) Cleanliness is essential when performing a venipuncture.

(2) It is most important that correct technique be practiced in order to avoid unnecessary pain to the patient, prevent tissue damage, secure a good representative blood specimen, and prevent contamination of the specimen or infection of the patient.

(3) Syringes and needles must be thoroughly inspected for damage or malfunction.

(4) If difficulty is experienced in entering the vein or a hematoma begins to form, release the tourniquet and promptly withdraw the needle and apply pressure to the wound.

(5) Vigorous pulling on the plunger of the syringe can collapse the vein, produce hemolysis of the blood specimen, or cause air to enter the syringe.

(6) When repeated venipunctures have to be performed on one patient, it is advisable to select different sites for blood withdrawal.

(7) Remove the tourniquet as early as possible once a good flow of blood has been established. Prolonged application of the tourniquet results in partial stasis of blood and changes many quantitative values of blood components.

(8) Blood drawn by venipuncture is often stored for a period of time before it is analyzed. For this reason, certain general precautions must be followed in order to insure a valid analysis. Before withdrawing blood from its container, make sure the blood sample is thoroughly but gently mixed. Blood containers should be tightly stoppered at all times to prevent drying or contamination. Store the blood specimen in the refrigerator. Blood counts should be done within 3 hours after collection. Under no circumstances should blood taken for hematological examinations be stored overnight.

3-3. Capillary Puncture:

a. Site. Several different sites are suitable for capillary puncture. Because it is the most accessible, the palmar or lateral surface of the tip of the finger (preferably ring finger) is the most common site in adults. However, certain problems can be encountered such as heavy calloused areas or excessive tissue fluids (edema) that tend to result in nonrepresentative samples. The lobe of the ear can be used for capillary puncture. However, differences in cell concentration do occur when blood is obtained from this site, primarily because of higher lymphocyte concentrations in the ear lobe. Because of the small amount of tissue on the fingers of infants, the preferred site is the heel or big toe. A modification of the normal technique that has proven quite satisfactory when working with the heel of infants is to make two incisions in a criss-cross fashion or "T."

NOTE: To be a valid report, work done on capillary blood must be from a FREE-FLOWING puncture wound.

b. Equipment:

(1) Gauze pads, 2 x 2 inches.

(2) Blood lancet.

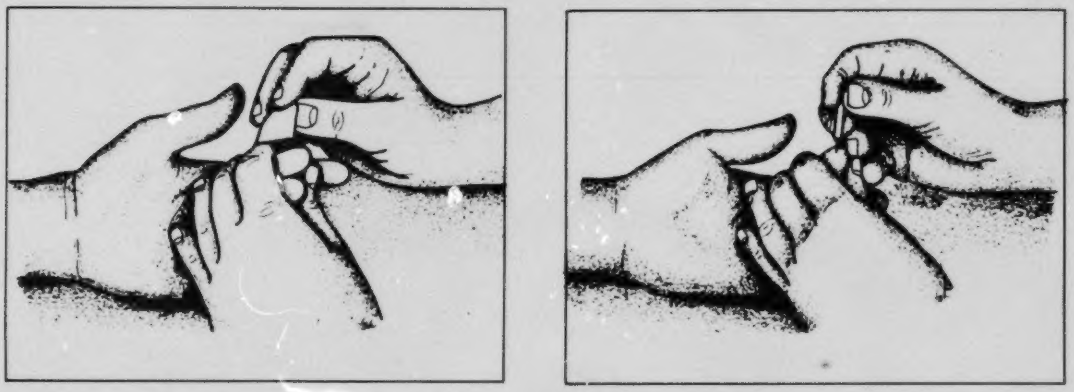
(3) Glass slides, heparinized capillary tubes, and other devices to receive the specimen.

(4) Isopropyl alcohol, 70 percent. Dilute 726 ml of 95 percent isopropyl alcohol to 1 liter with distilled water.

c. Procedure:

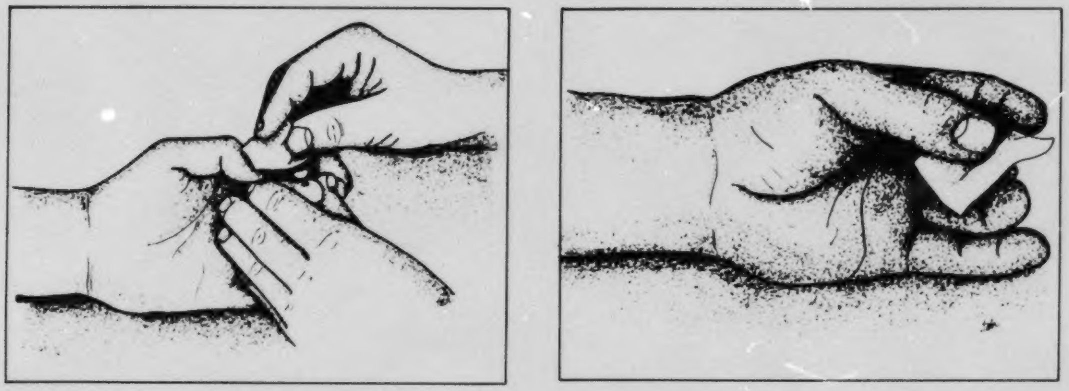
(1) The puncture site should be warm to assure good circulation of blood. If it is cold, apply warm water (38°–40° C) for a few minutes. If blood is to be drawn from the ear, the edge of the lobe, not the flat side, should be punctured.

(2) The site to be punctured is first rubbed with alcohol soaked gauze to remove dirt and epithelial debris, increase circulation, and render the area reasonably disinfected (figure 3–4a).



a. Clean the puncture site

b. Puncture the finger



c. Wipe away first drop of blood

d. Apply pressure to the site

Figure 3–4. Capillary Puncture Procedure.

(3) Allow sufficient time for the circulation to equalize.

(4) While making a finger puncture, apply gentle pressure to the finger to hold the skin taut. Hold the finger in one hand and the lancet in the other. The puncture is made perpendicular to the lines of the fingerprints, which results in a more free-flowing wound (see figure 3–4b).

(5) The first drop of blood that appears is wiped away before specimens are taken (see figure 3–4c).

(6) The blood must not be squeezed out since this dilutes it with fluid from the tissues, thus altering the ratio of cellular elements to fluid, as well as the ratio of cellular elements to each other.

(7) After the desired specimens have been collected, have the patient hold a sterile dry gauze pad over the wound until bleeding stops (see figure 3–4d).

d. Discussion:

(1) A disposable lancet is a very satisfactory instrument for puncture of the skin. However, if this is not available a sharp pointed surgical blade is quite suitable.

(2) Do not use the finger on a hand which has been hanging over the side of the bed as it is likely to be congested. Edematous or cyanotic areas should not be used.

(3) The finger should be thoroughly dry prior to puncture; blood will not well up on a finger that is moist. Furthermore, the alcohol or other antiseptic used can coagulate the blood proteins causing cell clumping and erroneous values as well as dilute cell volumes. This will result in incorrect counts and differentials.

(4) Finger punctures should be made along the lateral aspect of the fingertip. More nerve endings are located on the fingerprint area of the fingers, therefore, more pain results from punctures in this area. Scars can also form in these sensitive areas, and difficulty may be encountered in puncturing a callous. All of these difficulties are eliminated by drawing the blood from the lateral rather than the ventral aspect of the finger.

3–4. Anticoagulants:

a. Anticoagulants are used to prevent the coagulation of the blood specimens and the reagent employed should not bring about alteration of blood components. Unfortunately, many anticoagulants can alter cell structures as well as coagulation. The anticoagulants most often used are ethylenediamine-tetra-acetate (EDTA), ammonium-potassium oxalate (Heller and Paul double oxalate), and heparin.

b. The choice of anticoagulant will depend on the analysis to be made. Ethylenediamine-tetra-acetate (EDTA) is the anticoagulant of choice for most hematological analyses. This anticoagulant causes a minimum of distortion to the cells and platelets. It does not dissolve quickly in blood, however, so the tube must be inverted four or five times when blood is added. The dipotassium salt is prepared as a 1 percent solution in distilled water, and a final concentration of 0.5 ml of anticoagulant for each 5 ml blood is used. Another common anticoagulant is ammonium-potassium oxalate (Heller and Paul double oxalate). This combination of oxalates does not shrink or enlarge the red blood cells appreciably. It is essential, however, to add an optimal volume of blood to the oxalate, no less than 3.5 nor more than 6.0 ml.

c. Heparin does not alter the size of cellular components. It is, in fact, the standard for comparison of anticoagulant distortion. Heparin is more expensive and dissolves less readily than double oxalate salts. Approximately 0.5 to 1.0 mg is required to anticoagulate 5 ml of blood for 72 hours. The quantity of anticoagulant noted above in each case is sufficient to prevent clotting of the blood specimen. On the other hand, an excess of anticoagulant should be avoided because too much will result in distortion of cells and hemolysis. Ideally, differential blood smears should not be prepared from blood that contains an anticoagulant.

d. If oxalate is added to vials and dried in an oven, take great care to avoid temperatures above 80° C. Oxalates are converted to carbonates by prolonged exposure to elevated temperatures. Under normal circumstances, it should not be necessary to prepare your own oxalate solutions since prepared anticoagulant vacuum tubes are available from Federal medical supply sources.

e. A correctly anticoagulated blood sample is essential to the proper performance of a blood cell count. The cellular constituents must remain free in the plasma and should be as similar as is possible to those remaining in the patient's circulation.

SECTION B—PREPARATION AND STAINING OF BLOOD SMEARS

3-5. Introduction:

a. Invaluable information concerning a patient's condition can be gained from the examination of a blood smear. Slides and coverglasses must be perfectly clean and free of grease, oil, lint, or dust to prepare good blood smears. It is best to use new slides, but sometimes it is even necessary to clean new slides in 95 percent alcohol. There are two basic methods for the preparation of blood smears: the coverslip and slide methods. The coverslip method has certain advantages over the slide method; distribution of cells is more like that of the in vivo circulation. The principal disadvantage of the latter method is that coverslips are very fragile and easily broken during processing.

b. Many stains and techniques are used to stain blood films. Two types of stains are in general use—those which stain fixed cells, and those which will stain living cells (supra-vital stain). The panoptic (differentiating) stains generally used in hematology are Giemsa and Wright's stains. When optimal staining conditions exist, Wright's stain is very satisfactory and easily differentiates cells. Wright's stain powder is composed of eosin and methylene blue that stain selectively. The methylene blue is composed of several staining components. The eosin stains cell cytoplasm, and the methylene blue stains nuclear material, granules, and inclusions. Both stains oxidize rapidly because they are in alkaline solution. Giemsa, a purified polychrome stain, is added to compensate for this defect by maintaining the azurophilic staining property of the mixture.

3-6. Slide Method:

a. Principle. A small drop of blood is placed near one end of a clean glass slide. Using a second slide as the spreader, the blood is streaked into a thin film and allowed to dry. It is then fixed and stained with modified Wright's stain.

b. Equipment:

(1) Venipuncture or finger puncture material.

(2) Clean glass slides.

c. Reagents:

(1) Wright's Stain Buffer:

(a) Solution A: Dissolve 9.47 grams disodium phosphate (Na_2HPO_4) (dibasic), anhydrous, in a 1-liter volumetric flask containing about 750 ml of distilled water. Dilute to the mark with distilled water.

(b) Solution B: Dissolve 9.08 grams potassium acid phosphate (KH_2PO_4) (monobasic), anhydrous, in a 1-liter volumetric flask containing about 750 ml of distilled water. Dilute to the mark with distilled water.

(c) Mix 27 ml of the Na_2HPO_4 solution (Solution A) with 73 ml of the KH_2PO_4 solution (Solution B). Qs. to 1 liter. Final pH should be 6.4.

(2) Wright's Stain Solution:

(a) Add 9.0 grams of Wright's powder stain, 1.0 gram of Giemsa powder stain, and 90 ml of glycerin to a mortar of suitable size. Triturate this mixture thoroughly for 15–30 minutes.

(b) Transfer the mixture to a large open-mouth jar (a kitchen spoon is ideal for this purpose). Cover the jar and incubate the glycerin/stain mixture overnight at 37° C.

(c) Transfer the glycerin/stain mixture to a large brown bottle and add 2,910 ml of acetone-free methanol.

(d) Age the Wright's stain solution approximately 2 weeks in the dark. Mix daily to assure that the Wright's stain powder is completely dissolved.

d. Procedure:

(1) Make a finger puncture or venipuncture in the usual manner.

(2) Touch a drop of blood to a clean glass slide at a point midway between the sides of the slide and a short distance from one end. If a venipuncture is made, dispose the drop of blood directly from the needle. If a finger puncture is made, dispense the drop of blood from the puncture site after discarding the first drop.

NOTE: The drop of blood should be no larger than 1/8 to 3/16 inch in diameter (see figure 3-5a).

(3) Lay the specimen slide on a flat surface and hold it securely. Place a smooth, clean edge of the spreader slide on the specimen slide at an angle of about 30° from the horizontal (see figure 3-5a).

(4) Pull the spreader slide toward the drop of blood until contact is made within the acute angle formed by the two slides as shown in figure 3-5b.

(5) Allow the blood to spread toward the sides of the slide.

(6) Push the spreader slide smoothly and lightly toward the opposite end of the specimen slide, drawing the blood behind it into a thin film (see figure 3-5c).

(7) Allow the blood film to air-dry completely. Do not blow on the slide in an effort to enhance drying.

(8) Using a lead pencil, write the name (or identification) of the patient in the thick area of the smear. Do not use a wax pencil as it dissolves during the staining process.

(9) Cover the slide completely with Wright's stain and allow it to remain on the smear for about 2 minutes to fix the blood cells. The stain should cover the slide but should not be allowed to overflow the edges; the stain must be replenished should it begin to evaporate.

(10) Add an equal volume of Wright's stain buffer directly to the stain and blow the mixture gently to assure maximum mixing. Allow it to remain for about 4 minutes.

NOTE: The times recommended for staining and buffering are approximate and should be adjusted with each fresh batch of stain to give the most satisfactory results.

(11) Using tap water, float off the mixture of stain and diluent from the slide to avoid the deposition of metallic scum on the smear. The scum appears after the addition of the buffer to the stain. Wash the slide thoroughly under cold, slowly-running water.

(12) Air dry the smear and wipe the excess stain from the under surface of the slide.

e. Discussion:

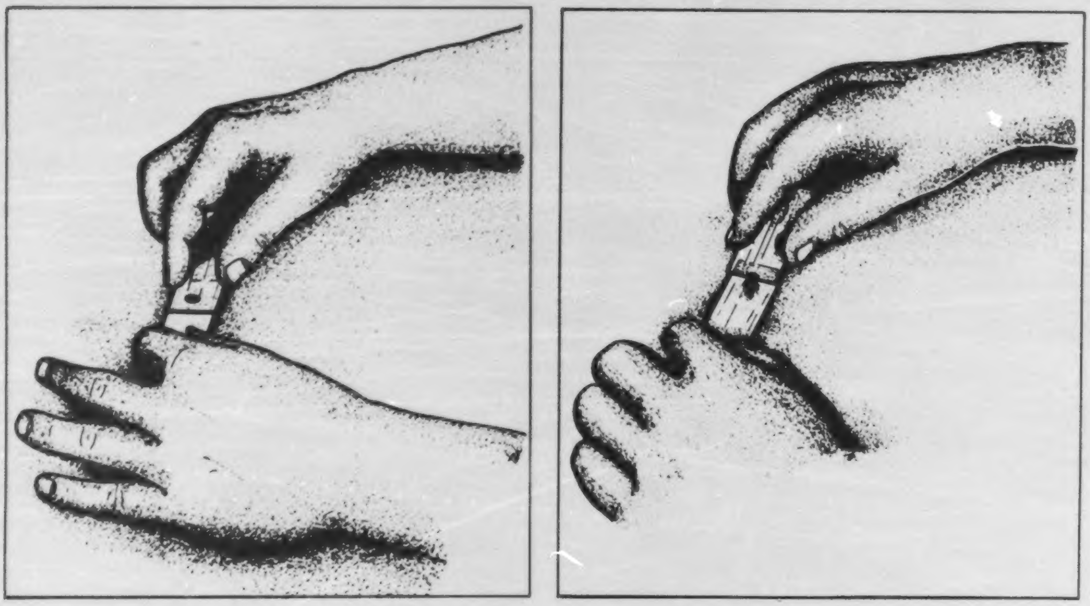
(1) A properly prepared blood smear is margin-free; has no lines, ridges, or holes; is placed centrally on the slide; has an adequate thin area; and has a uniform distribution of leukocytes.

(2) It is preferable that blood smears not be made from blood containing anticoagulants since the leukocytes change their staining characteristics, develop vacuoles, engulf oxalate crystals, and show nuclear deformities. However, satisfactory slides are made with blood anticoagulated with EDTA.

(3) Avoid the following errors:

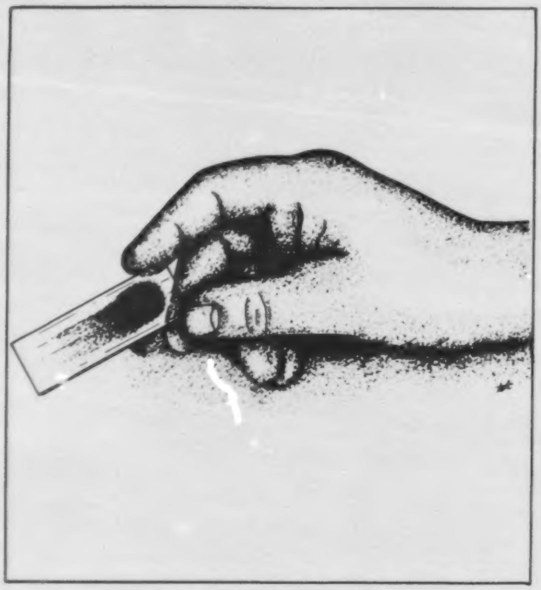
(a) Thick films made from an excess amount of blood placed on the slide.

(b) Delay in transferring the blood to the slide.



a. Place spreader slide at an angle of about 30° from the horizontal

b. Contact the blood with the spreader slide



c. Push spreader slide to the opposite end of the slide drawing the blood behind it into a thin film

Figure 3-5. Slide Method for Preparation of Blood Films.

(c) A spreader slide that has damaged or unpolished ends.

(d) The use of dirty, dusty, greasy, or scratched slides.

(4) If slides cannot be stained immediately, they should be dried and then fixed in methyl alcohol for 30 minutes.

(5) In cases of marked leukopenia, smears can be prepared from the white cell layer ("buffy coat") obtained by centrifuging the blood slowly in a Wintrobe hematocrit tube at 500–800 rpm for 5 minutes.

(6) It is important that the blood film be completely dried before staining; otherwise the wet areas will wash off the slide.

(7) Protect blood slides from insects such as flies, cockroaches, etc. They can "clean" raw blood slides very rapidly.

(8) Protect slides from areas of high humidity. Excessive moisture tends to hemolyze red blood cells.

(9) Slides should be stained as soon as possible after preparation. White cells tend to become distorted and to disintegrate very rapidly, thus causing considerable difficulty in identification.

(10) Very little actual staining takes place during the fixation stage. Most of the staining actually occurs during the buffering stage.

(11) During the buffering stage, it is important that only amounts of buffer equal to the stain be added, otherwise there is a tendency to overdilute, causing the smear to stain weakly.

(12) After the staining is complete, do not blot the smear but air-dry it. To speed up the drying process, the smear can be placed in the heat of the substage light. It is important that the slide not be heated too intensely or too long since overheating tends to darken the staining reaction.

(13) A good quality smear should macroscopically be buff in hue. It should not be blue, green, or red. Microscopically, the red blood cells should be a light orange and the white blood cells bluish if they display their true staining color.

(14) If the RBCs are bluish or green, this indicates that the stain is too alkaline. With an alkaline stain, the WBCs stain heavily and generally display fair distinguishing characteristics. However, any abnormalities of the RBCs are masked by the heavy stain. Heavy staining can be caused by:

(a) Blood smears which are too thick.

(b) Overstaining (prolonged buffer action).

(c) Evaporation of the methanol in the stain.

(d) Stain or diluent which is alkaline.

(e) Alkaline fumes.

(15) If the red blood cells are bright red, the stain is too acid. In this condition they stain well but the white blood cells (except eosinophilic granules) stain very poorly if at all. Thus, the stain is of no value for differential studies. Tendency toward acid staining is caused by:

(a) Incomplete drying before staining.

(b) Insufficient staining (insufficient buffer action).

(c) Overdilution of the stain with buffer.

(d) Prolonged washing of the slide after staining.

(e) Stain or buffer which is acid.

(f) Acid fumes.

(16) The staining reactions of blood are as follows:

Type of blood cell or component	*Good stain*	*Acid stain*	*Alkaline stain*
Erythrocytes	Buff pink	Bright red	Blue or green
All nuclei	Purple-blue	Pale blue	Dark blue
Eosinophilic granules	Granules red	Brilliant red, distinct	Deep gray or blue
Neutrophilic granules	Violet-pink	Pale	Dark, prominent
Lymphocyte cytoplasm	Blue	Pale blue	Gray or lavender

(17) The technician should strive for a staining reaction which is neither too alkaline nor too acid. Such a stain gives good distinguishing features for all the cells of the blood system.

(18) If the staining reaction is excessively alkaline, this can be corrected by decreasing time of staining or neutralizing the stock stain solution with 1 percent acetic acid or 1 percent hydrochloric acid. Add the acid a drop of a time. Check the results after the addition of each drop of acid with trial slides.

(19) If the staining reaction is excessively acid, this may be corrected by increasing the time of staining or neutralizing the stock stain solution with 1 percent potassium bicarbonate or a weak solution of ammonia water. Add the ammonia water or potassium bicarbonate one drop at a time. Check the results on trial slides after the addition of each drop of the neutralizer.

(20) Staining reactions can also be varied by adjusting the proportions of disodium phosphate and potassium acid phosphate used in preparing the buffer. For example, increasing the proportion of disodium phosphate will make the buffer more alkaline; reducing it will make the buffer more acid.

(21) A poorly stained smear can sometimes be saved by washing rapidly with 95 percent alcohol, washing quickly in water, then restaining.

(22) Automatic staining devices (see chapter 2) are currently being used in many USAF hospitals.

3-7. Coverglass Method:

a. Principle. A blood specimen is spread in a uniform even layer between two coverslips by placing a drop of blood on one coverglass, placing a second coverglass upon it, and deftly pulling the coverglasses apart.

b. Equipment:

(1) Capillary or venipuncture material.

(2) Coverglasses, No. 2, 22 x 22 mm.

c. Reagents:

(1) Wright's Stain Buffer. See paragraph 3-6c.

(2) Wright's Stain Solution. See paragraph 3-6c.

d. Procedure:

(1) Prepare patient for capillary puncture or venipuncture, and perform the puncture.

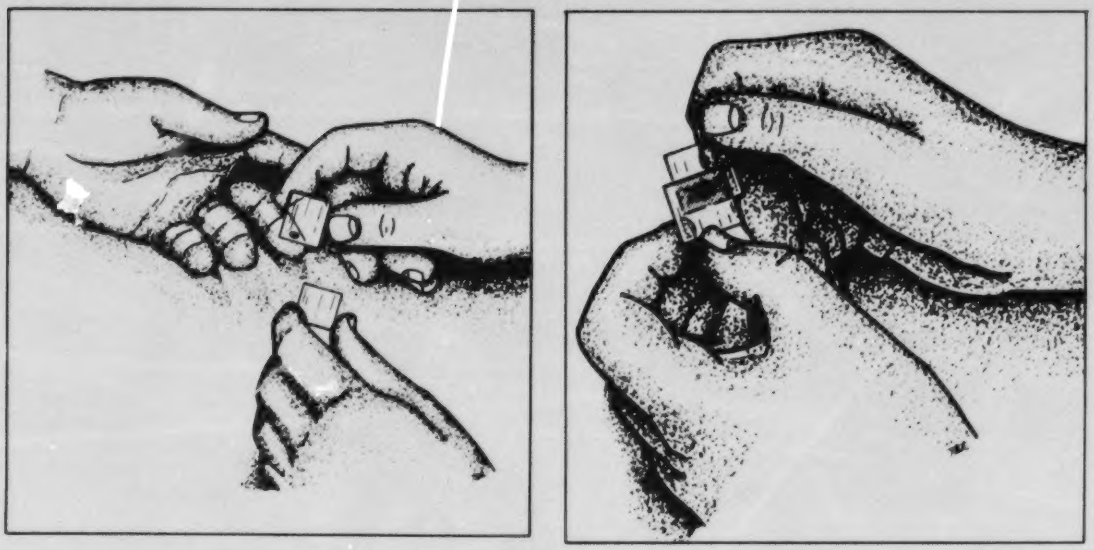
(2) Without touching the skin, bring a coverslip into contact with the blood specimen (figure 3-6a). If the venipuncture technique is employed, dispense a drop of blood directly from the needle onto the coverslip.

(3) Quickly place another coverslip over the drop of blood (see figure 3-6b). Let the blood spread until it has almost stopped spreading.

(4) Pull the two coverslips apart in sliding fashion. As the coverglasses are pulled apart they must be held in an absolutely horizontal axis (see figure 3-6c). Any tendency to a vertical pull ruins the preparation, since holes in the smear may result.

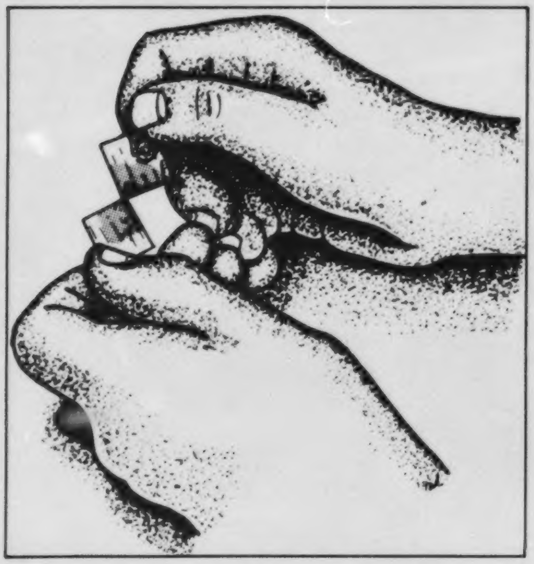
(5) Place the preparation (smear side up) on a clean paper and allow to dry in the air.

(6) Stain and examine according to the method described for the slide method above.



a. Bring a cover slip into contact with the blood specimen

b. Place another cover slip over the drop of blood



c. Pull the two cover glasses apart in a sliding fashion

Figure 3-6. Coverglass Method for Preparation of Blood Films.

e. Discussion:

(1) The coverglass method gives a much better distribution of white cells and is the method preferred by many in making differential white counts.

(2) The slide method renders satisfactory differential counting difficult because the larger white cells are pushed to the edges and the lymphocytes remain scattered through the spread.

(3) When red cells are to be examined for parasites, the slide method is superior, inasmuch as more fields are available for study and red cell distribution is as good as with the coverglass method.

(4) Some disadvantages of the coverglass method are that coverglasses are awkward to handle, easily broken, and must be mounted on a glass slide before they can be examined.

SECTION C—BONE MARROW SPECIMENS

3-8. Introduction. Examination of the bone marrow is an important diagnostic tool when a disease affecting the hematopoietic system is present or suspected. Bone marrow aspiration or biopsy is always performed by a physician. In most cases the laboratory technician will assist the physician by preparing the bone marrow material for examination by a pathologist.

3-9. Processing of Bone Marrow Specimens for Examination:

a. Principle. Several bone marrow samples are smeared on a series of slides, stained, and examined microscopically for cytological detail. The procedure outlined is to be modified as required.

b. Reagents:

(1) Wright's Stain Buffer. See paragraph 3-6c.

(2) Wright's Stain Solution. See paragraph 3-6c.

(3) Formalin Fixative, 10%. Dilute 10 ml of 40% formaldehyde (R.G.) to 100 ml with distilled water.

c. Procedure:

(1) From the first aspirate of marrow (approximately 0.5 ml) prepare several films on coverglasses or slides.

(a) Quickly place a drop of marrow on the end of a slide or coverglass.

(b) Remove excess blood with a capillary pipet or a syringe leaving the gray marrow particles and a small amount of blood behind.

(c) Prepare thin films by the slide or coverglass method.

(d) Optional slides are made at this time for various staining procedures.

(2) Imprint slides are prepared by placing a drop of aspirated marrow on a slide or coverglass. The particles are picked out and transferred to another slide or coverglass. The particles are crushed gently and smeared.

(3) The clot remaining in the syringe is placed in a wide mouth jar containing the formalin fixative solution for histological examination.

(4) The physician may obtain a second aspirate of marrow (approximately 1 ml). This is added to a small tube containing EDTA and centrifuged in a Wintrobe hematocrit tube at 2500 rpm for 8 minutes. Aspirate off the plasma. Remix the plasma and all of the buffy cellular layer and prepare smears of the mixture.

(5) Stain the required slides with Wright's stain. Slides should be fixed in methyl alcohol if a delay is experienced before staining.

(6) Process other slides for additional stains as required.

(7) In certain cases the marrow aspiration is performed for bacteriologic diagnosis. Express the aspirate into a sterile tube containing 1 ml of 1 percent sodium citrate. Bacteriologic examination can be made as required.

d. Discussion:

(1) A properly prepared bone marrow smear should meet the following criteria:

(a) Thin smear 3 to 5 cm in length and 2 cm wide.

(b) Nucleated cells almost touching.

(c) Visible fatty areas and high concentration of marrow foci.

(2) The procedure described above is an outline, and deviations from it should be in accordance with instructions from the pathologist.

(3) All slides must be accurately labeled.

(4) They should be air-dried immediately.

(5) As soon as they are thoroughly dry, bone marrow slides should be fixed in methanol to prevent disintegration of the more fragile cells.

(6) Since interpretation of findings in bone marrow examinations is very difficult, even when performed by trained persons, it is of utmost importance that the smears and stains be carefully prepared using scrupulously clean equipment.

Chapter 4

MORPHOLOGY OF BLOOD CELLS

SECTION A—GENERAL INFORMATION

4-1. Basic Concepts of Cell Morphology:

a. In this chapter all normal and most commonly seen abnormal blood cells are morphologically described. Although general rules for identification are given along with representative photographs and drawings, it is important to realize that no biological entity fits the guidelines precisely.

b. The present classification of blood cells is man's attempt at identifying stages of maturation by assigning artificial steps to a continuing process. The process is a smooth, continuing one, and therefore no one cell ever precisely fits the criteria for a specific stage. These stages are artificial classifications which exist to simplify identification.

4-2. General Rules of Cell Identification. Certain general rules are applied to all cell maturation (hemopoiesis), either in the erythrocyte, leukocyte, thrombocyte, or plasmocyte series. Although these rules are broken by individual cells, they are an aid to classifying cells.

a. Immature cells are larger than mature cells and become smaller as they mature.

b. The relative and absolute size of the nucleus decreases as the cell matures. In some cell series the nucleus disappears.

c. The cytoplasm in an immature cell is quite blue in color and lightens as the cell matures.

d. The young nucleus is reddish and becomes bluer as the cell ages.

e. Nuclear chromatin is fine and lacy in the immature cell. It becomes coarse and clumped in the more mature cells.

f. If there is doubt in the identity of a cell, classify to the more mature form.

SECTION B—ERYTHROCYTES

4-3. Introduction:

a. In the normal development and maturation (erythropoiesis) of the erythrocytic series, the red blood cell undergoes a graduation of morphological changes. This cell development is a gradual transition and six different stages can be identified. The nomenclature used to describe red blood cells is recommended by the American Society of Clinical Pathologists and the American Medical Association. The terms with some of their synonyms are as follows:

ASCP Terminology	*Synonyms*
Rubriblast	Pronormoblast
Prorubricyte	Basophilic normoblast
Rubricyte	Polychromatophilic normoblast
Metarubricyte	Orthochromatic normoblast
Diffusely basophilic erythrocyte	Polychromatic erythrocyte
Erythrocyte	Normocyte

b. Erythropoiesis is regulated by the intake of substances to build the cells, the storage of these substances, and their proper utilization. When normal erythropoiesis occurs, both the cytoplasm and the nuclei of the cells grow at a synchronized rate. Individual differences in physiology and physical structure of the erythrocyte account for minor morphological changes so often encountered. In certain diseases, these morphological changes may vary to a greater extent. These variations occur in size, shape, staining, and inclusions in the erythrocyte.

4-4. Erythrocytic Series. The erythrocytic series is illustrated in figure 4-1.

a. Rubriblast:

(1) Size: 14 to 19 microns in diameter.

(2) Nucleus: This cell has a large round to oval purple nucleus which occupies most of the cell. The nuclear chromatin is arranged in a close mesh network forming a reticular appearance. There are 0-2 light blue nucleoli present within the nucleus.

(3) Cytoplasm: The cytoplasm is dark blue, granule-free, and limited to a thin rim around the nucleus. There is no evidence of hemoglobin formation.

b. Prorubricyte:

(1) Size: 10 to 15 microns in diameter.

(2) Nucleus: The nucleus is generally round, dark purple, and smaller than the nucleus of the rubriblast. The chromatin is coarse and clumped giving the nucleus a darker stain. Nucleoli are usually not present, but when they are, they appear more prominent than in the rubriblast.

(3) Cytoplasm: The cytoplasm is royal blue and more abundant than in the rubriblast. Cytoplasmic granules are not present.

c. Rubricyte:

(1) Size: 8 to 10 microns in diameter.

(2) Nucleus: The nucleus is dark, round or oval, and smaller than the prorubricyte nucleus. The chromatin material is found in dense, irregular clumps. Nucleoli are not present.

(3) Cytoplasm: The cytoplasm is more abundant than in the precursor cells. It is blue-pink (polychromatic), the pink resulting from the first visible appearance of hemoglobin. Cytoplasmic granules are absent.

d. Metarubricyte:

(1) Size: 7 to 10 microns in diameter.

(2) Nucleus: This cell has a small, round, and blue-black degenerated nucleus. The nuclear chromatin is clumped and uniformly dense.

(3) Cytoplasm: The cytoplasm appears as a wide band around the nucleus. It is orange-red due to the hemoglobin content. Cytoplasmic granules are absent.

e. Diffusely Basophilic Erythrocyte:

(1) Size: 7 to 9 microns in diameter.

(2) Nucleus: the nucleus is absent.

(3) Cytoplasm: The cytoplasm stains a bluish buff with Wright's stain and there is no central light pallor as in the erythrocyte. With supravital staining this cell will show light blue reticulum strands in the cytoplasm and is called a reticulocyte.

f. Erythrocyte:

(1) Size: 6 to 8 microns in diameter.

(2) Nucleus: The nucleus is absent.

(3) Cytoplasm: The cytoplasm of the periphery is light orange with a central zone of pallor. The appearance of the central zone of pallor is due to the biconcave morphology of the cell, which allows more light through the center than through the margin areas.

4-5. Variations in Erythrocytes. These variations are illustrated in figure 4-2.

a. Size:

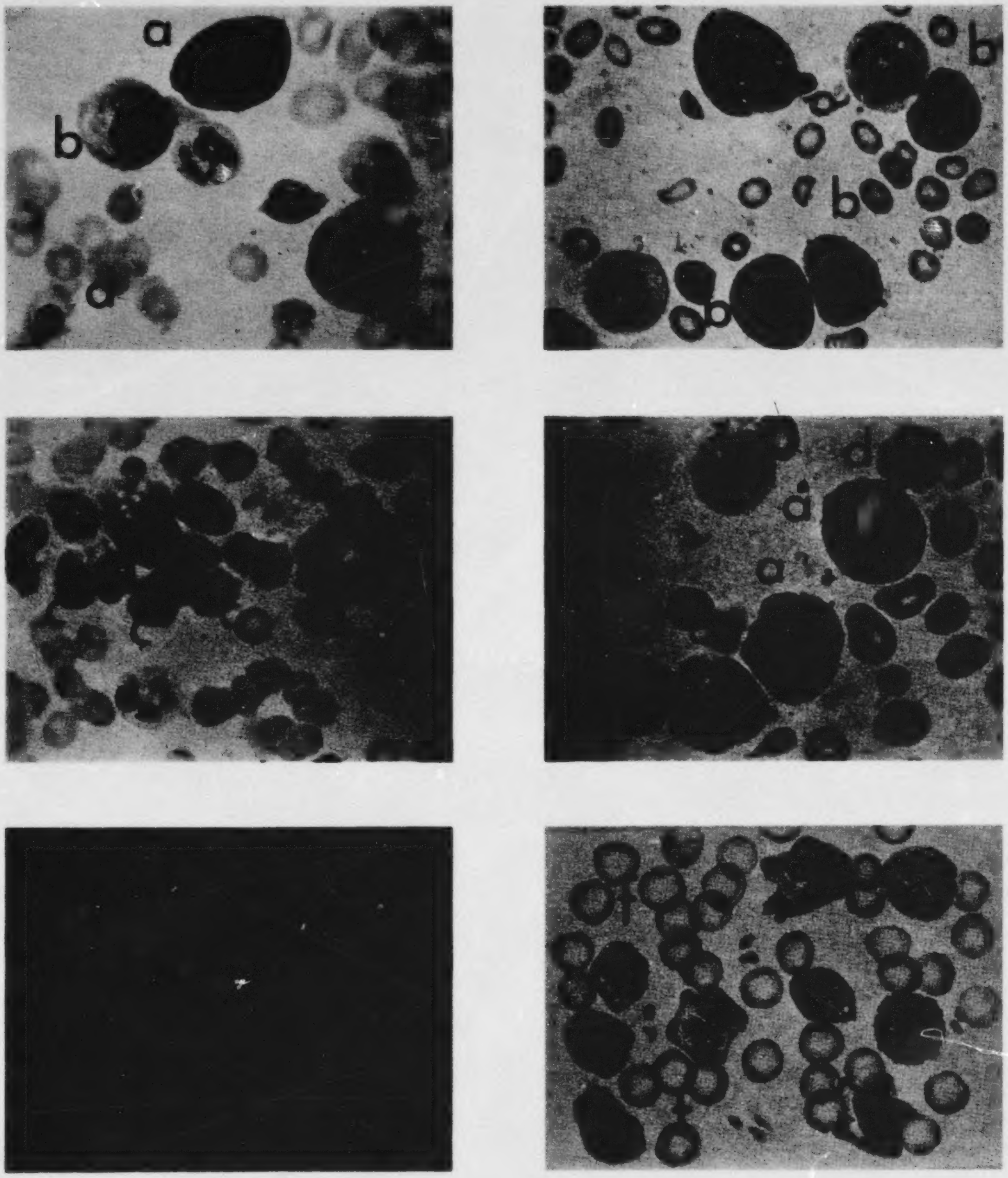
(1) Anisocytosis. Anisocytosis is a variation in the size of erythrocytes beyond the normal limits. Cells of varying size are seen in the same fields. It is a frequent finding in leukemia, pernicious anemia, and other anemias.

(2) Macrocytes. Macrocytes are erythrocytes larger than 9 microns in diameter. These cells can be found in healthy newborn infants, pernicious anemia, and other anemias.

(3) Microcytes. Microcytes are erthrocytes smaller than 6 microns in diameter. These cells are frequently seen in iron deficiency, spherocytic, and Mediterranean hemolytic anemia.

b. Shape:

(1) Poikilocytosis. This term describes a marked variation in the shape of erythrocytes. Poikilocytes can be pear-shaped,



a. Rubriblast
b. Prorubricyte
c. Rubricyte
d. Metarubricyte
e. Reticulocyte
f. Erythrocyte

Figure 4-1. Erythrocytic Series.

comma-shaped, oval-shaped, or various other bizarre forms. These cells are encountered in pernicious anemia and many other types of anemia.

(2) Sickle Cell (Drepanocytes). Sickle cells are abnormal erythrocytes which assume a crescent or sickle-shaped appearance under conditions of reduced oxygen tension. The presence of sickle cells is an inherited abnormality due to the presence of hemoglobin S. Sickle cell anemia is encountered primarily in Negroes.

(3) Spherocytes. These are abnormal erythrocytes which are spherical in shape, having a diameter smaller than normal, and a darker stain (without central pallor) than normal erythrocytes. These cells are found in the hemolytic diseases and are particularly characteristic of congenital hemolytic jaundice, a hereditary disorder.

(4) Ovalocytes (Elliptocytes). These cells are abnormal erythrocytes which have an oval or "sausage" shape. Ovalocytosis is inherited and can be seen in thalassemia and sickle cell anemia.

(5) Target Cells (Leptocytes). Target cells are erythrocytes which have deeply stained (pink) centers and borders, separated by a pale ring, giving them a target-like appearance. These cells have a greater than normal diameter. The cells can be seen in various types of hemolytic anemias, in liver disease, and after a splenectomy.

(6) Burr Cells. Burr cells are triangular or crescent-shaped erythrocytes with one or more spiny projections on the periphery. These are seen in small numbers in uremia, carcinoma of the stomach, and peptic ulcer.

(7) Acanthrocytes. Acanthrocytes are irregularly-shaped erythrocytes with long spiny projections. This is a congenital abnormality associated with low serum concentration of low density (beta) lipoproteins.

(8) Crenated Erythrocytes. These cells have serrated or prickly outlines resulting from shrinkage of the cells. This condition occurs when blood films dry too slowly and the surrounding plasma becomes hypertonic. This deformity is artificial and has no pathological significance except when crenated cells are found in spinal fluid as discussed in the chapter concerned with performing cell counts on cerebrospinal fluid.

(9) Schistocytes. These cells are fragments of erythrocytes. Frequently these cells have a hemispherical shape (helmet cells). Schistocytes can be found in severely burned patients and many anemias.

(10) Rouleaux Formation. This phenomenon is adherence of erythrocytes to one another presenting a stack-of-coins appearance. It occurs in conditions characterized by increased amounts of fibrinogen and globulin.

c. Staining:

(1) Hypochromia. Hypochromia is a condition in which the normal central pallor is increased due to decreased hemoglobin content. This condition is characteristic of many anemias.

(2) Polychromatophilia. This term describes nonnucleated erythrocytes which show bluish coloration instead of light orange. Polychromatophilia is due to the fact that the cytoplasm of these cells does not mature, resulting in the abnormal persistence of the basophilic cytoplasm of the earlier nucleated stages.

d. Inclusions:

(1) Howell-Jolly Bodies. These are nuclear remnants found in the erythrocytes of the blood in various anemias. They are round, dark violet granules about 1 micron in diameter. Generally, only one Howell-Jolly body will be found in any one red cell. However, two or more may sometimes be present. Howell-Jolly bodies generally indicate absent or nonfunctioning spleen.

(2) Cabot's Rings (Ring Bodies). These are bluish threadlike rings found in the red cells in the blood of patients with severe anemias. They are interpreted as remnants of the nuclear membrane and appear as ring or "figure-eight" structures. Usually only one such structure will be found in any one red cell.

(3) Basophilic Stippling. Round, small, blue-purple granules of varying size in the cytoplasm of the red cell represent a condensation of the immature basophilic substance (see polychromatophilia) which normally disappears with maturity. This is known as basophilic stippling. It can be demonstrated by standard staining techniques in contrast to reticulocyte filaments which require a special stain. Stippling occurs in anemias and heavy metal poisoning (lead, zinc, silver, mercury, bismuth) and denotes immaturity of the cell.

(4) Heinz-Ehrlich Bodies. These are small inclusions found primarily in those hemolytic anemias induced by toxins. They are round, refractile bodies inside the erythrocyte and are visible only in unfixed smears. It is thought that they are proteins which have been denatured and that they are an indication of erythrocyte injury.

(5) Siderocytes. These are erythrocytes containing iron deposits. These deposits indicate an incomplete reduction of the iron from ferric to the ferrous state which is normally found in hemoglobin. Prussian blue stain must be used to readily demonstrate these cells.

e. Megaloblastic Erythrocytes. The development of megaloblastic cells is caused by a deficiency of vitamin B_{12} or folic acid. Pernicious anemia is a disease considered to be due to a deficiency in vitamin B_{12} and/or certain related growth factors. With this deficiency the erythrocytes do not mature normally and are generally larger than normal. The most notable characteristic of this abnormal maturation is a difference in the rates of maturation of the cytoplasm and the nucleus. The development of the nucleus is slower than that of the cytoplasm, so that in the more mature of the nucleated forms a spongy nucleus as well as an exceptionally large size may be observed. Nuclear chromatin in the megaloblast is much finer and is without the clumps observed in the rubriblast. Such development is termed asynchronism. The mature cell is large (about 10 microns) and is termed a megalocyte. The younger cells of this series are named by adding the suffix "pernicious anemia type," that is, rubricyte, pernicious anemia type, etc.

SECTION C—LEUKOCYTES

4-6. Granulocytic Series. The stages in the normal maturation of the granulocytes are: myeloblast, promyelocyte, myelocyte (neutrophilic, eosinophilic, and basophilic), metamyelocyte (neutrophilic, eosinophilic, and basophilic), band cell (neutrophilic, eosinophilic, and basophilic), and segmented cell (neutrophilic, eosinophilic, and basophilic). As the granulocytes mature, the granules increase in number. These granules later become specific and differ in the affinity for various dyes. Neutrophilic granules do not stain intensely with either dye. Basophilic granules have an affinity for the basic or blue dye. Eosinophilic stain red with an affinity for the acid dye. The criteria for identification of the various stages of the granulocytic series are: size of cell, nucleus-cytoplasm ratio, nuclear shape, number of nucleoli, and the type and size of cytoplasmic granulation. The granulocytic series is illustrated in figure 4-3.

a. Myeloblast:

(1) Size: 12 to 18 microns in diameter.

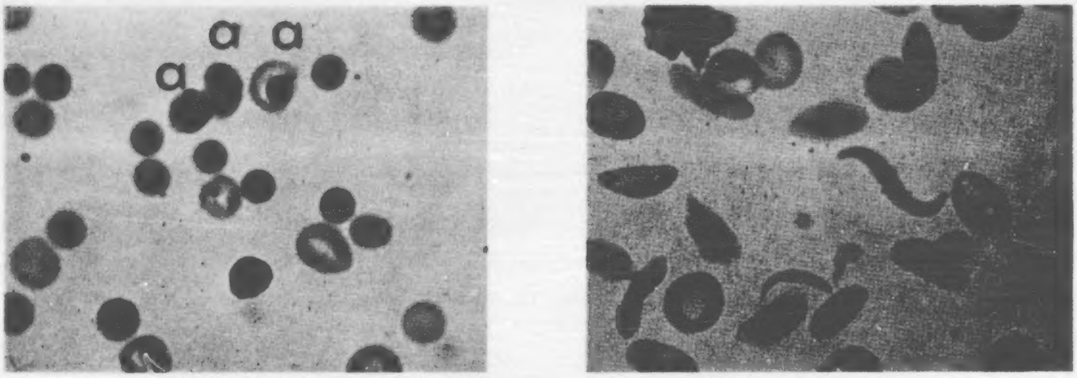
(2) Nucleus: The nucleus is round or ovoid and stains predominantly reddish-purple. The interlaced chromatin strands are delicate, well-defined, and evenly stained. Two or more pale blue nucleoli are demonstrable. The nucleus occupies most of the cell with a nucleus-cytoplasm ratio of 6:1. It is separated from the cytoplasm by a definite nuclear membrane.

(3) Cytoplasm: The cytoplasm is a narrow, deep blue, nongranular rim around the nucleus.

b. Promyelocyte:

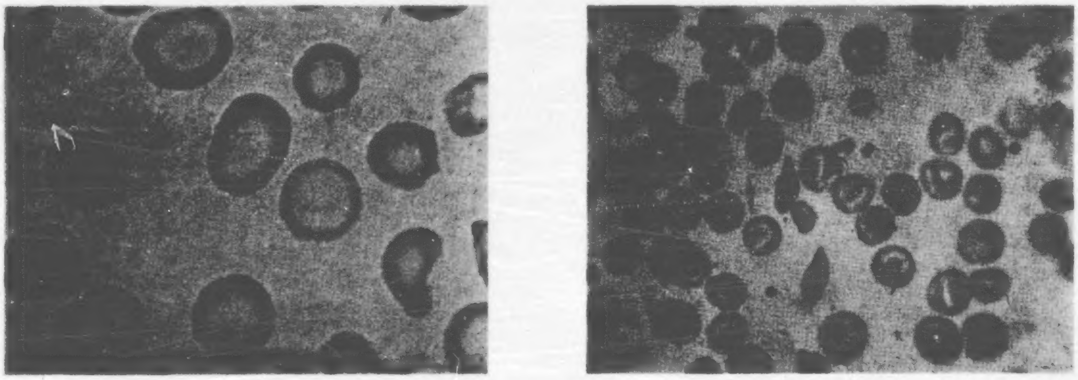
(1) Size: 12 to 20 microns in diameter.

(2) Nucleus: The nucleus is round or ovoid with coarse-clumping, purple chroma-



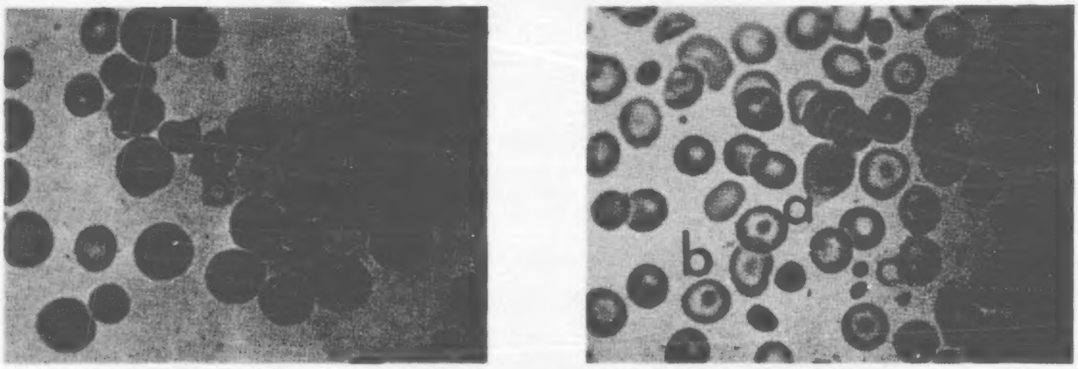
a. Metarubricytes Anisocytosis
Polychromasia Spherocytosis

Poikilocytosis: Sickle Cells



Hypochromic Macrocytic Erythrocytes

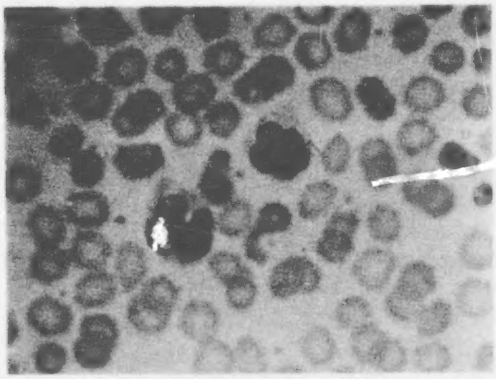
Marked Poikilocytosis
Anisocytosis and Target Cells



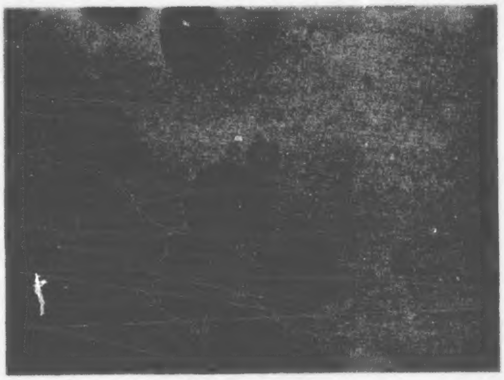
a. Metarubricyte
b. Howell—Jolly Bodies

a. Metarubricyte
b. Target Cell
c. Crenated RBC

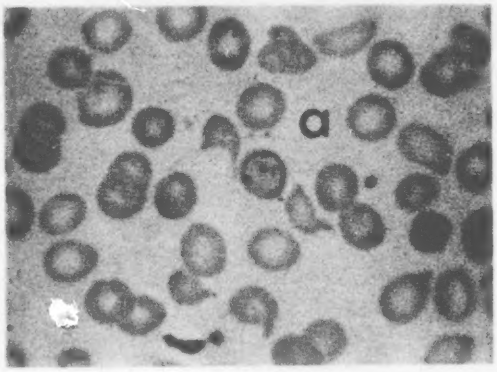
Figure 4-2. Variations in Erythrocytes.



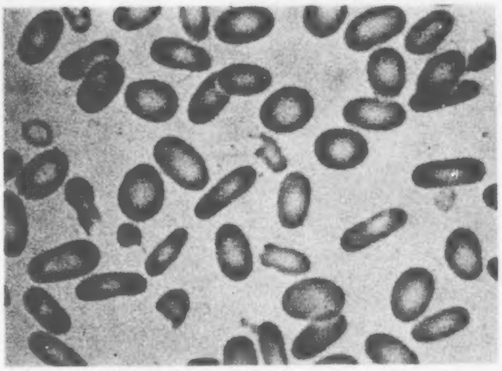
Crenated RBC Burr Cells
Acanthocytes 2 Leukocytes



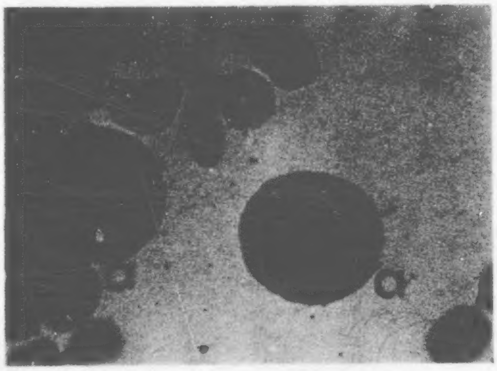
a. Basophilic Stippled Erythrocyte



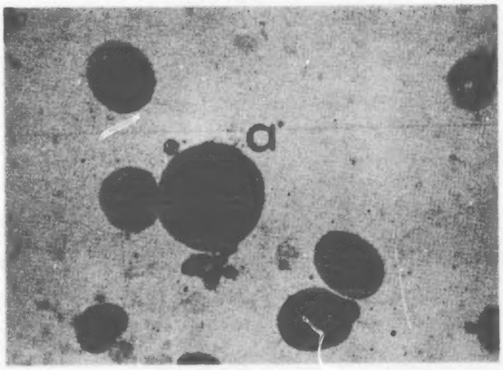
a. Cabot Ring



Elliptocytes (Oval Erythrocytes)



a. Rubricytes (Pernicious Anemia)



a. Metarubricyte
(Pernicious Anemia)

Figure 4-2. Continued.

tin material. One to three oval, light-blue nucleoli are usually present. The nucleoli are less distinct than in the myeloblast. This cell has a nucleus-cytoplasm ratio of 4:1.

(3) Cytoplasm: The cytoplasm is light purple and contains varying numbers and sizes of dark nonspecific granules which stain red to purplish-blue. The granules usually overlie the nucleus.

c. Myelocyte. In the myelocytic stage the granules are definite and so numerous that frequently they obscure nuclear detail. While promyelocytes are sometimes distinguished as neutrophilic, eosinophilic, or basophilic, the differentiation is generally considered as first occurring in the myelocytic stage.

d. Neutrophilic Myelocyte:

(1) Size: 12 to 18 microns in diameter.

(2) Nucleus: The nucleus is round, oval, or flattened on one side. The chromatin strands are light purple, unevenly-stained, and thickened. Nucleoli are usually absent. The nucleus is smaller than the earlier cells of this series with a nucleus-cytoplasm ratio of 2:1.

(3) Cytoplasm: The cytoplasm is bluish-pink and contains a small relatively light area of ill-defined, pink granules which develop among the dark, nonspecific, azurophilic granules of the promyelocyte. As the myelocyte ages, the dark granules become less prominent and the light-pink-colored neutrophilic granules predominate.

e. Neutrophilic Metamyelocyte:

(1) Size: 10 to 18 microns in diameter.

(2) Nucleus: The nucleus is indented or kidney shaped. The nuclear chromatin stains dark purple and is condensed into irregular strands. Nucleoli are absent. The nucleus-cytoplasm ratio is approximately 1.5:1.

(3) Cytoplasm: The cytoplasm is pinkish-blue and covered with many small, light pink granules.

f. Neutrophilic Band:

(1) Size: 10 to 16 microns in diameter.

(2) Nucleus: The nucleus is shaped like a horseshoe with a dark pyknotic mass at each pole of the nucleus where the lobes develop. The nucleus-cytoplasm ratio is approximately 1:2.

(3) Cytoplasm: The cytoplasm contains many small, evenly distributed light pink granules.

g. Neutrophilic Segmented Cell:

(1) Size: 10 to 15 microns in diameter.

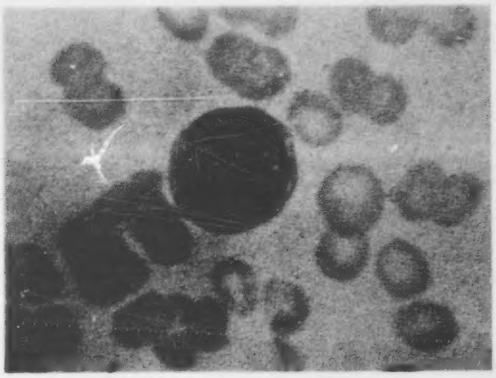
(2) Nucleus: The nucleus has definite lobes separated by a very narrow filament or strand. The nucleus-cytoplasm ratio is approximately 1:3.

(3) Cytoplasm: The cytoplasm is light pink and the small, numerous, and evenly distributed neutrophilic granules have a light pink color.

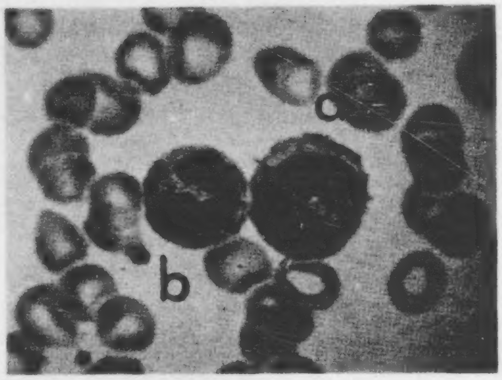
h. Development of the Eosinophilic Group. Cells of the eosinophil group are characterized by relatively large, spherical, cytoplasmic granules which have a particular affinity for the eosin stain. The earliest eosinophil (myelocyte) has a few dark spherical granules with reddish tints which develop among the dark, nonspecific granules. As the eosinophilic cells pass through their various developmental stages, these granules become less purplish-red and more reddish-orange. The dark blue, nonspecific granules, characteristic of the promyelocyte and the early myelocyte stages, disappear. Because the percentage of eosinophils is usually low in bone marrow or peripheral blood smears, no useful clinical purpose is served by routinely separating the eosinophils into their various myelocyte, metamyelocyte, band, and segmented categories. On the other hand, in situations such as eosinophilic leukemia in which the eosinophils are greatly increased, an analysis of the incidence of the various stages would be useful in diagnosis.

i. Mature Eosinophil:

(1) Size: 10 to 15 microns in diameter.

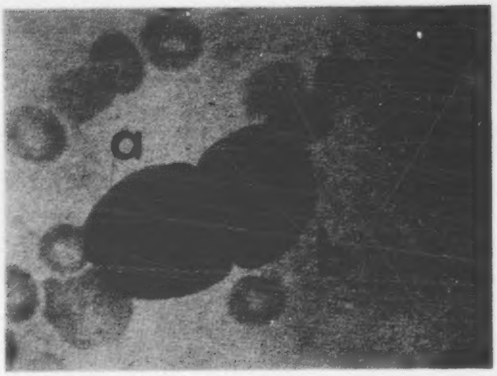


Myeloblast



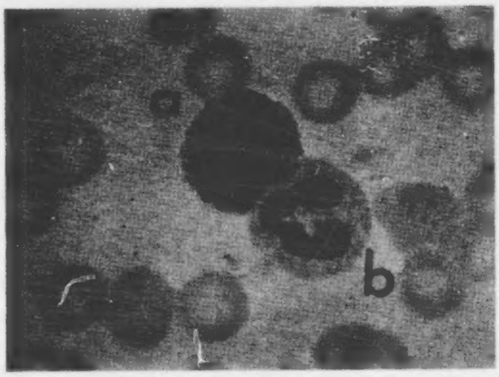
a. Metamyelocyte

b. Band Neutrophil



a. Promyelocyte

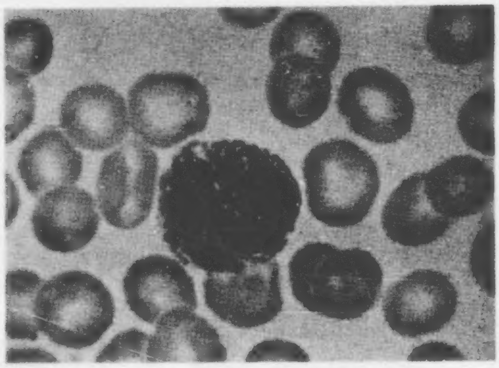
b. Promyelocyte with Auer Body



a. Basophil

b. Neutrophil: Segmented

Myelocyte



Eosinophil

Figure 4-3. Granulocytic Series.

(2) Nucleus: The nucleus has definite lobes separated by a very narrow filament or strand. Seldom does an eosinophil have more than two lobes.

(3) Cytoplasm: The cytoplasm contains bright reddish-orange, distinct granules. The granules are spherical, uniform in size, and evenly distributed throughout the cytoplasm, but rarely overlie the nucleus.

j. Development of the Basophilic Group. These cells have round, indented, band, or lobulated nuclei and are classified according to the shape of the nuclei, as basophilic myelocytes, metamyelocytes, bands, and segmented forms. These cells are so few in peripheral blood and bone marrow that there is little clinical value in differentiation of the various maturation stages.

k. Mature Basophils:

(1) Size: 10 to 15 microns in diameter.

(2) Nucleus: The nucleus has definite lobes separated by a very narrow filament or strand. The nuclear details are obscured by the cytoplasmic granulation.

(3) Cytoplasm: The cytoplasm is covered by many blue to black granules. These granules are unevenly distributed and vary in number, size, shape, and color.

4-7. Agranulocytes. Agranulocytes are leukocytes devoid of specific granulation. These cells generally originate in the lymphatic system, but can be found in normal bone marrow. Agranulocytes include the lymphocytic series, monocytic series, and plasmocytic series.

4-8. Lymphocytic Series. The stages in the development of the lymphocytic series are: lymphoblast, prolymphocyte, and lymphocyte. These cells are fragile and can show shape variants. Lymphocytes usually have round contours, blue cytoplasm, and eccentrically located round nuclei. Cells of this series are differentiated on the basis of the nuclear chromatin. The lymphocytic series is illustrated in figure 4-4.

a. Lymphoblast:

(1) Size: 10 to 18 microns in diameter.

(2) Nucleus: The nucleus has an oval or round shape and stains reddish-purple. The nuclear chromatin is fine, well distributed, and coarser than in the myeloblast. Chromatin is condensed at the edges of the nucleus to form a definite nuclear membrane. One or more nucleoli are present. The nucleus is prominent with a nucleus-cytoplasm ratio of 6:1.

(3) Cytoplasm: The cytoplasm is deep blue with a frequent perinuclear clear zone.

b. Prolymphocyte:

(1) Size: 10 to 18 microns in diameter.

(2) Nucleus: The nucleus is oval and slightly indented. The nuclear chromatin is coarse, slightly clumped, and dark purple. One light blue nucleolus is usually present. The nucleus-cytoplasm ratio is 5:1.

(3) Cytoplasm: The cytoplasm varies from light blue to dark blue and it can show a few red-purple (azurophilic) granules.

c. Lymphocyte:

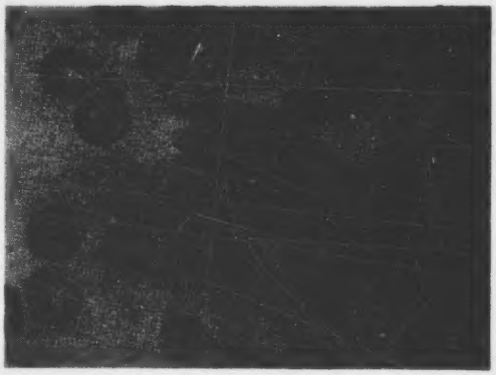
(1) Size: The mature cell of this series varies greatly in size. Small lymphocytes are 7 to 9 microns in diameter. The large lymphocytes are 10 to 18 microns in diameter.

(2) Nucleus: The nucleus is round or oval and can be slightly indented. The nuclear chromatin is markedly condensed, dark purple-blue, and clumped. Nucleoli are absent and a definite nuclear membrane is present. The nucleus-cytoplasm ratio is approximately 1.5:1.0.

(3) Cytoplasm: The cytoplasm is light blue to blue with a perinuclear clear zone around the nucleus. A few azurophilic granules can be seen in the cytoplasm of larger lymphocytes.

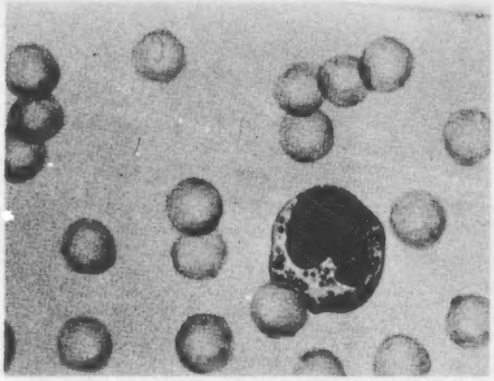
4-9. Monocytic Series. The stages in the development of the monocytic series are monoblast, promonocyte, and monocyte. Cells of

a. Lymphoblast
b. Lymphocyte
c. Smudge Cell



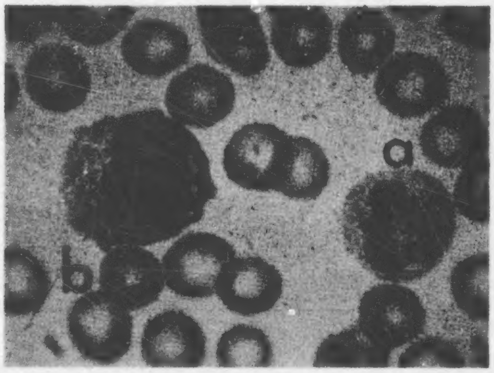
a. Monoblast
b. Stem (Ferrata Cell)

Prolymphocyte



Promonocyte

Lymphocyte:
Azurophilic Granulation



a. Neutrophil (Late Band)
b. Monocyte

Figure 4-4. Lymphocytic Series and Monocytic Series.

the series are slightly larger than granulocytes. They are round with smooth margins and seldom show shape variants. The mature monocyte is differentiated from the lymphocyte and metamyelocyte by the very fine, light staining nucleus. The monocytic series is illustrated in figure 4-4.

a. Monoblast:

(1) Size: 12 to 20 microns in diameter.

(2) Nucleus: The nucleus is round or oval and red-purple in color. The nuclear chromatin is fine and well distributed. One to two nucleoli are present. The nucleus-cytoplasm ratio is 6:1.

(3) Cytoplasm: The cytoplasm is a clear, deep blue and forms a thin rim around the nucleus.

b. Promonocyte:

(1) Size: 12 to 20 microns in diameter.

(2) Nucleus: The nucleus is irregularly-shaped and light purple. The nuclear chromatin is fine and spongy. A nucleolus can be present. The nucleus-cytoplasm ratio is 5:1.

(3) Cytoplasm: The cytoplasm is gray-blue with fine red-purple (azurophilic) globules.

c. Monocyte:

(1) Size: 12 to 16 microns in diameter.

(2) Nucleus: The nucleus is round or kidney-shaped, but can be deeply indented or have two or more lobes. One of the most distinctive features of the monocyte is the presence of superimposed lobes, giving the nucleus the appearance of brainlike convolutions. Heavy lines marking the edges of the folds and grooves are features which are not seen in other cells. Another feature of the nucleus, which is of value in diagnosis, is the tendency for the nuclear chromatin to be loose with light spaces in between the chromatin strands, giving a coarse linear pattern in contrast to the lymphocyte which has clumped chromatin. Nucleoli are absent. The nucleus-cytoplasm ratio is approximately 3:1.

(3) Cytoplasm: The cytoplasm of the monocyte is dull gray-blue while the cytoplasm of the neutrophils in the adjacent fields is definitely lighter and is pink rather than gray-blue. The nonspecific granules of the monocyte are usually fine and evenly distributed, giving to the cell a dull, opaque or ground-glass appearance. In addition to the background of evenly distributed nonspecific granules, there may be a few unevenly distributed larger azurophilic granules. Vacuoles are often demonstrable in the cytoplasm.

4-10. Plasmocytic Series. Plasmocytes constitute approximately 1 percent of the white cells in the normal bone marrow. These cells can be present in the peripheral blood in chronic infections, granulomatous and allergic diseases, and multiple myeloma. The stages of development are: plasmoblast, proplasmocyte, and plasmocyte. These cells are illustrated in figure 4-5.

a. Plasmoblast:

(1) Size: 14 to 24 microns in diameter.

(2) Nucleus: The nucleus is large, oval or round, and contains within the fine, purplish, reticulated chromatin one to three blue nucleoli. The nucleus-cytoplasm ratio varies from 2:1 to 1:1.

(3) Cytoplasm: The cytoplasm is relatively abundant and stains dark blue. No granules or vacuoles are found in the cytoplasm.

b. Proplasmocyte:

(1) Size: 14 to 22 microns in diameter.

(2) Nucleus: The nucleus is ovoid and located eccentrically. The chromatin is purple, coarser, and more clumped. One to two nucleoli are present. The nucleus-cytoplasm ratio is 2:1.

(3) Cytoplasm: The cytoplasm is pale blue, with pale reddish-blue vacuolation. A pale perinuclear zone or "halo" is usually present.

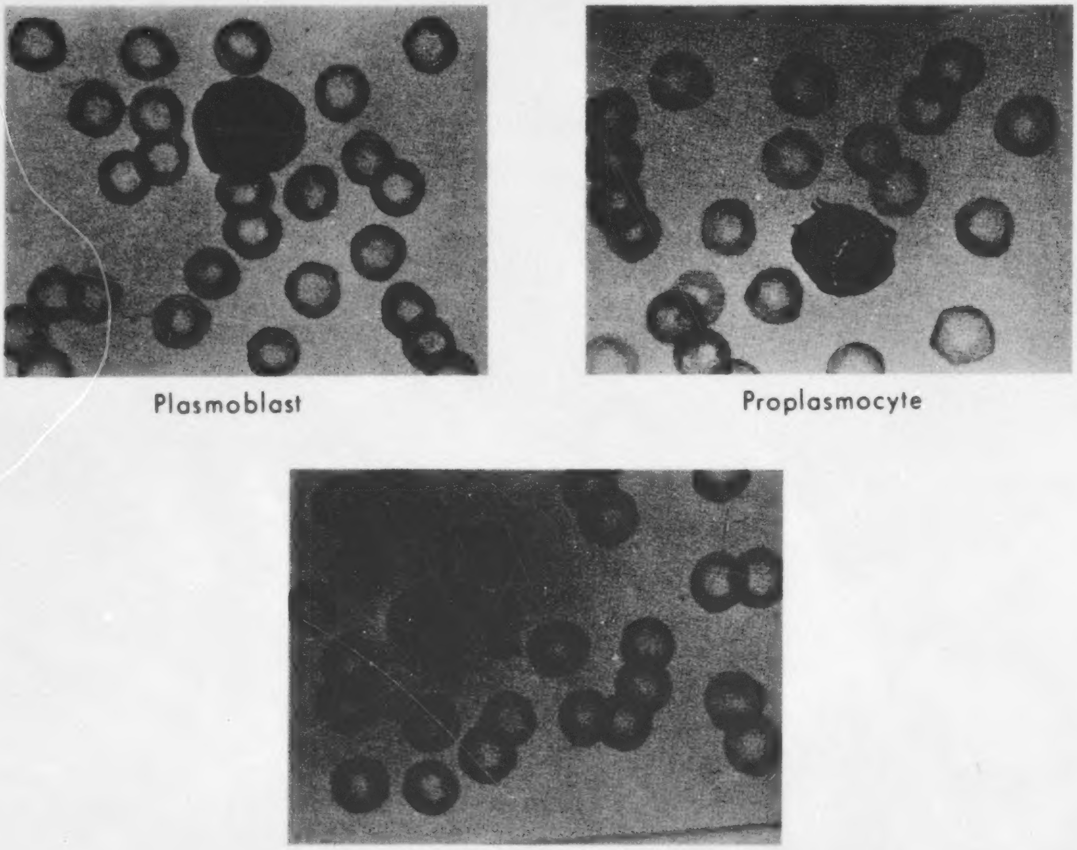
c. Plasmocyte:

(1) Size: 8 to 18 microns in diameter.

(2) Nucleus: The nucleus is small, ovoid, and eccentrically located. The chromatin is coarse, lumpy, and purple. Nucleoli are not usually present. The nucleus-cytoplasm ratio is 1:2.

(3) Cytoplasm: The cytoplasm adjacent to the nucleus is lightly stained in contrast to the periphery of the cell which has a high saturation of red and blue dyes. In some cells the dark cytoplasm has a greenish or larkspur-blue color. The cytoplasm contains multiple small and relatively unstained globules embedded in a bluish-red filamentous matrix. It is the presence of these tapiocalike globules in the dark surrounding medium which gives the plasmocyte its characteristic mottled and foamy appearance and its brilliant translucency. In occasional cells the globules can be quite prominent and take a red or bluish-red stain. Such globules are called Russell or fuchsin bodies, or eosinophilic globules. Vacuoles of various sizes are frequently demonstrable.

4-11. Variations of Leukocytes. Variations of leukocytes occur as a result of abnormal maturation of the nucleus and/or cytoplasm. These variations are induced by leukemic states, infectious diseases, and toxicity. Many variations in leukocytes have been observed; however, this manual describes only the most frequently-occurring variations. A more complete study of leukocyte variations can be obtained from the references listed in the bibliography of this manual.



Plasmoblast

Proplasmocyte

Plasmocyte

Figure 4-5. Plasmocytic Series.

Variations in leukocytes are illustrated in figure 4-6.

a. Dohle Bodies. Dohle bodies are light blue or blue-gray, small, round inclusions found in the cytoplasm of neutrophilic leukocytes. This variation may occur in toxic conditions such as severe infections, burns, and scarlet fever.

b. Auer Rods. Auer rods are rod or spindle-shaped, cytoplasmic inclusions. They stain red-purple and are 1 to 6 microns long and less than 1.5 microns thick. They are frequently found in leukemias.

c. Toxic Granulation. Toxic granulation occurs in the neutrophilic metamyelocyte, band, and segmented cells. These granules are distinguished from the normal granulation because they are coarser and stain a dark purple. The variations occur in toxic states and severe infection.

d. Basket Cell. A basket cell is a ruptured leukocyte which has a network appearance. These cells result from a partial breakdown of the immature and fragile leukocytes. Basket cells are found predominantly in diseases with an acute shift toward immature forms, for example, leukemias.

e. Vacuolated Cell. A vacuolated cell is a degenerated cell with holes or vacuoles in the cytoplasm. Vacuolated cells can be seen in severe infections, poisoning, and leukemias, and in cells that have been in Heller & Paul oxalate too long.

f. Hypersegmentation. A normal neutrophilic segmented cell has a nucleus with an average of three lobes or segments. In a hypersegmented cell the nucleus is broken up into five to ten lobes or segments. This cell usually has a larger diameter than a normal neutrophilic segmented cell. Hypersegmentation is often seen in pernicious anemia.

g. Atypical Lymphocytes (Virocytes, Turk Cells). These lymphocytes are characteristic of infectious mononucleosis but they may also be seen in apparently healthy individuals and those with certain other diseases. Atypical lymphocytes are larger than normal and vary in appearance. Downey and McKinley described three types of atypical lymphocytes, but this classification has no real clinical purpose.

(1) Size: Large, up to 20 microns in diameter.

(2) Nucleus: The nucleus is oval or kidney shaped with very coarse chromatin strands not as lumpy as a normal lymphocyte.

(3) Cytoplasm: The cytoplasm is blue to dark blue. Often it is vacuolated which gives rise to a foamy appearance.

h. L.E. Cells:

(1) Persons having lupus erythematosus, one of the "collagen" diseases, have an abnormal plasma protein which causes swelling and breakdown of certain blood cell nuclei in vitro. This degenerated nuclear material attracts phagocytic cells, particularly segmented neutrophils, which engulf this nuclear mass. The resulting phagocyte and inclusion material is termed an "L.E." cell.

(2) The nucleus of an L.E. cell is adjacent to the peripheral outline of the inclusion material. The inclusion is smooth and smoky or light purple and has no visible chromatin network.

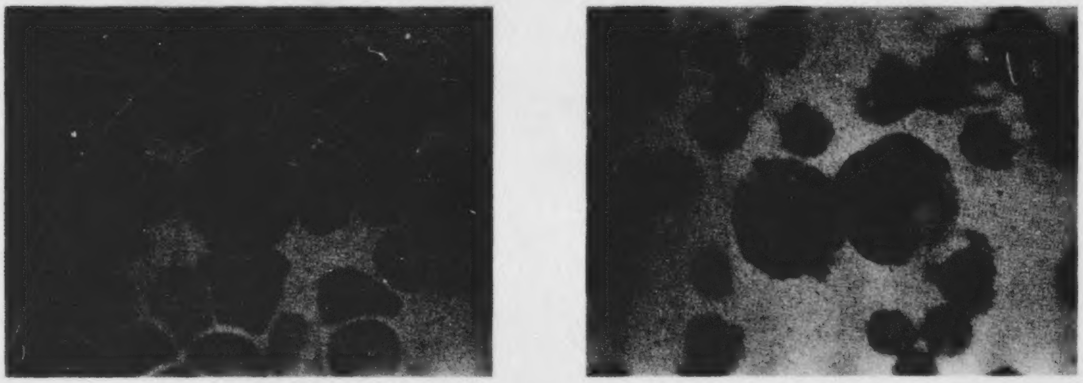
i. Rosettes. Rosette formation is the intermediate stage in the formation of an L.E. cell. A rosette formation consists of neutrophilic leukocytes surrounding free masses of lysed nuclear material.

j. Tart Cells. A tart cell contains lysed nuclear material within its cytoplasm. It differs from an L.E. cell because the inclusion retains characteristic nuclear structure. This inclusion is not smooth and has a darker staining periphery.

SECTION D—THROMBOCYTES

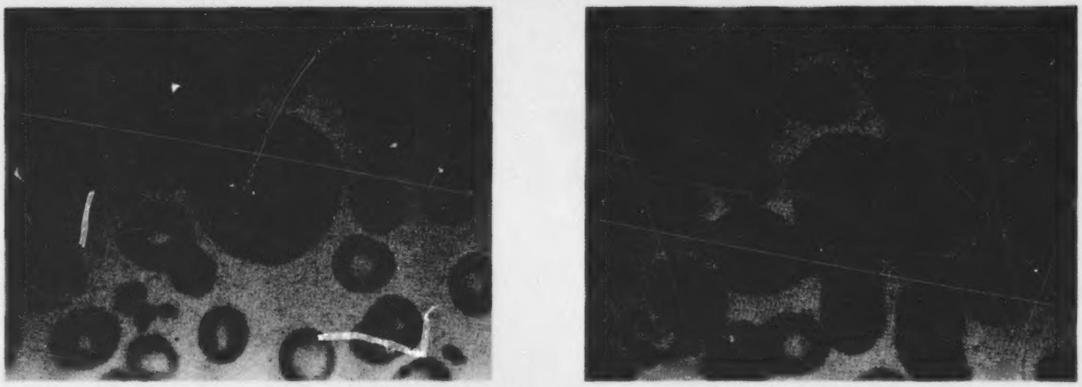
4-12. Introduction:

a. The general pattern of thrombocyte maturation is slightly different from that of leukocyte maturation. The cells of the megakaryocytic series tend to grow larger as they mature until there is cytoplasmic frag-



Band Neutrophil: Toxic Granulation

L.E. Cells



Neutrophil: Hypersegmented

Atypical Lymphocytes: Infectious Mononucleosis

Figure 4-6. Variations in Leukocytes.

mentation (or breaking off) to form the cytoplasmic thrombocytes seen in the peripheral blood.

b. Azurophilic granulation begins to appear in the second stage of development and continues until it almost obscures the nuclear lobes. The nucleus develops from a fine single lobe to multiple ill-defined lobes. The stages in the normal maturation of the megakaryocytic series are: megakaryoblast, promegakaryocyte, megakaryocyte, metamegakaryocyte, and thrombocyte. These cells are illustrated in figure 4-7.

4-13. Megakaryocytic Series:

a. Megakaryoblast:

(1) Size: 15 to 30 microns in diameter.

(2) Nucleus: One to two large oval or kidney-shaped nuclei are present. The reddish nuclear chromatin has a granular appearance. Several nucleoli are present within the nucleus but these are difficult to see. The nucleus-cytoplasm ratio is approximately 10:1.

(3) Cytoplasm: The cytoplasm is blue,

nongranular, and may show blunt protrusion.

b. Promegakaryocyte:

(1) Size: 40 to 100 microns in diameter.

(2) Nucleus: One to two indented round or oval nuclei are present. The nuclear chromatin is purple, coarse, and granular. Nucleoli are present but indistinct

(3) Cytoplasm: The cytoplasm is blue and has blunt, bubbly extensions. Azurophilic granules are usually present.

c. Megakaryocyte:

(1) Size: 40 to 100 microns in diameter.

(2) Nucleus: Two to sixteen nuclei are present and these are superimposed on and attached to each other by narrow filaments. Nucleoli are indistinct. Nuclear chromatin is lumpy and coarsely linear.

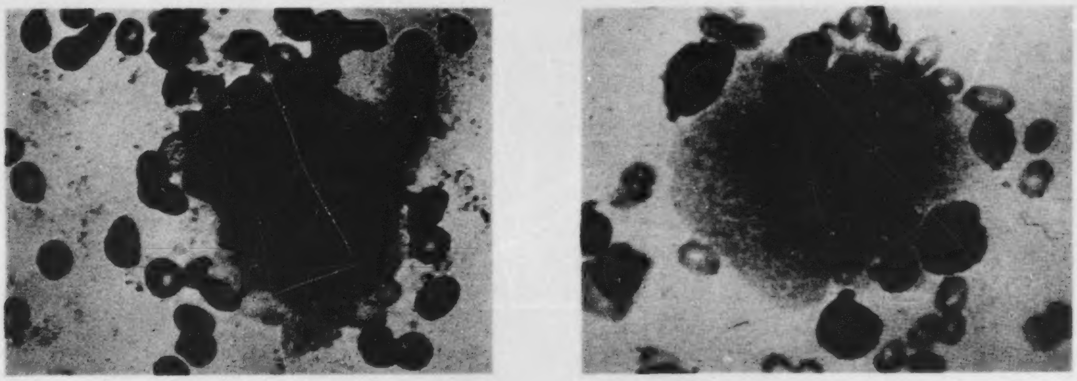
(3) Cytoplasm: The cytoplasm is covered with numerous, small blue-purple granules. No evidence of platelet fragmentation is present.

d. Metamegakaryocyte:

(1) Size: 40 to 100 microns in diameter.

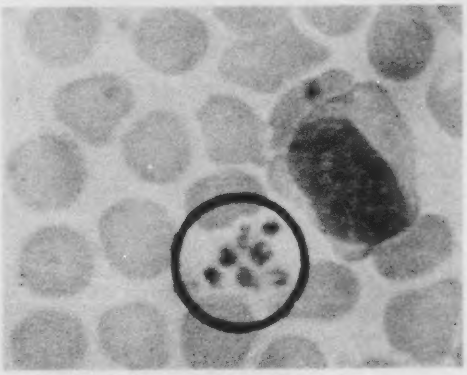
(2) Nucleus: Same as megakaryocyte.

(3) Cytoplasm: The cytoplasm is much like the megakaryocyte but shows evidence



Megakaryoblast: Bone Marrow

Megakaryocyte



Thrombocytes

Figure 4-7. Megakaryocytic Series.

of fragmentation or sloughing of thrombocytes from the cytoplasmic margins.

e. Thrombocytes:

(1) Size: 1 to 3 microns in diameter.

(2) Nucleus: None.

(3) Cytoplasm: The cytoplasm appears light blue with centrally located azurophilic (red-purple) granules. NOTE: Normally, megakaryocytes and metamegakaryocytes do not appear in the peripheral blood; however, they can be seen in some leukemias. In bone smears, there should be little difficulty in recognizing cells as being of this series because of their enormous size.

Chapter 5

METHODS IN HEMATOLOGY

SECTION A—MANUAL CELL COUNTS

5–1. Introduction:

a. Blood cells are subject to quantitative variations as well as the qualitative variations described in chapter 4. Some diseases stimulate the production of blood cells while others prevent or diminish the production of blood cells. For this reason a cell count gives valuable information to the physician concerning his patient's condition. Furthermore, in the case of the leukocyte count, the total count is necessary to calculate absolute counts for each type of leukocyte. This is done by multiplying the total count by the percentage of the particular cell type.

b. Cell counts can be performed by a variety of methods. Erythrocytes and leukocytes are counted by manual methods or automated methods. Other cell counts are performed only by manual methods. It is important when performing a cell count to maintain good quality control. Great care should be taken when performing any cell count.

c. The following paragraphs outline procedures for red blood cell (RBC) count, white blood cell (WBC) count, total eosinophil count, absolute basophil count, reticulocyte count, cerebrospinal fluid (CSF) cell count, and sperm count. The RBC and WBC counts are routinely done; they are performed either by the hemacytometer methods (manually) or by automated methods. Total eosinophil and absolute basophil counts are performed by a hemacytometer method using special diluting fluids to accentuate these cells. Reticulocytes are demonstrated by using a supravital stain. Semen analysis and CSF counts are included in this section because a hemacytometer is used to perform the appropriate counts.

5–2. Red Blood Cell Count (Hemacytometer):

a. Principle. A sample of blood is diluted with a special isotonic solution in a micropipet. After adequate mixing, a portion of the sample is introduced into the counting chamber (hemacytometer). The red blood cells (erythrocytes) in a known volume are then counted.

b. Reagents. The most common diluting fluids used for the RBC count are Gower's solution and Hayem's solution. Either solution can be used and the preparation of both is given below. Normalsaline may also be used.

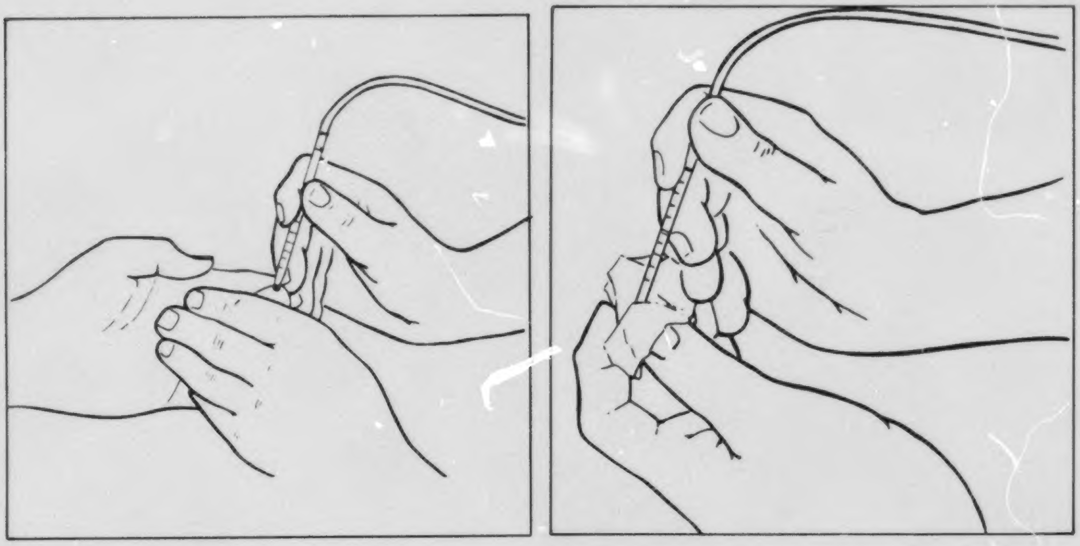
(1) Gower's Diluting Fluid. Add 12.5 grams of sodium sulfate (Na_2SO_4), anhydrous, and 33.3 ml glacial acetic acid to a 200-ml volumetric flask. Dilute to the mark with distilled water. NOTE: Add the water slowly and mix thoroughly.

(2) Hayem's Diluting Fluid. Add 0.5 gram mercuric chloride ($HgCl_2$), 5.0 grams sodium sulfate (Na_2SO_4), and 1.0 gram sodium chloride (NaCl) to a 200-ml volumetric flask. Dissolve the contents with distilled water and dilute to the mark with distilled water. Filter the solution several times through the same filter paper. NOTE: Deterioration of this solution can occur after three weeks. This solution must be discarded if a precipitate forms.

c. Procedure:

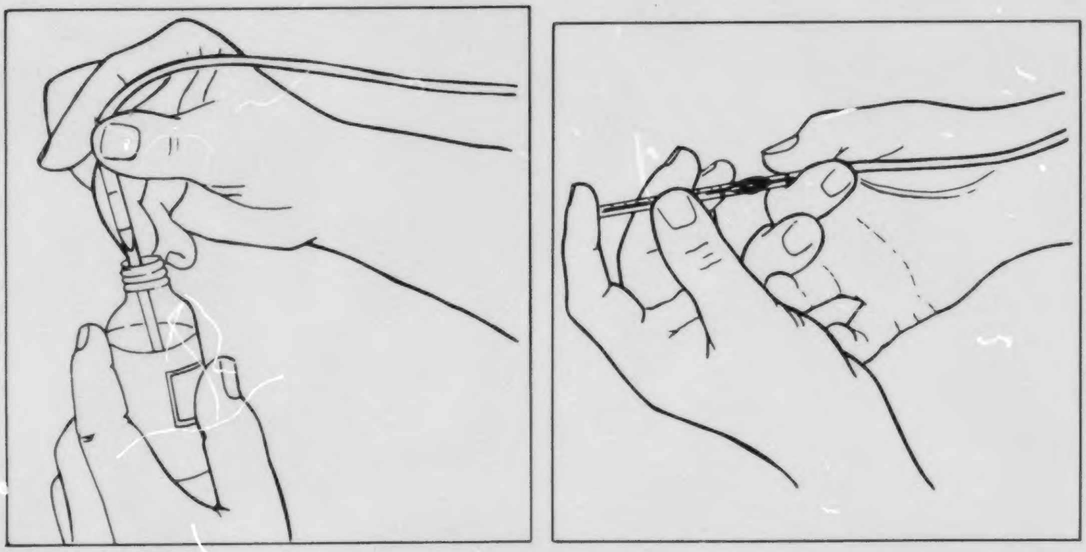
(1) Draw well-mixed capillary or venous blood to the 0.5 mark on the red blood cell diluting pipet. The blood column must be free of bubbles. (See figure 5–1a.)

(2) Wipe off the excess blood from the outside of the pipet to avoid transfer of cells to the diluting fluid. When wiping, take care not to touch the end of the pipet with gauze. (See figure 5–1b.)



a. Draw blood to appropriate mark on the pipet tube

b. Wipe off excess blood adhering to the tip of the pipet



c. Insert pipet tip into appropriate type of diluting fluid and draw up fluid to proper marking on pipet tube

d. Rotate pipet in a "figure-eight" motion

Figure 5-1. Processing Blood for Manual Cell Counts.

(3) Immediately draw diluting fluid to the 101 mark, at the same time rotating the pipet between the thumb and forefinger to mix the specimen and diluent. Hold the pipet upright to prevent air bubbles from flowing into the bulb (see figure 5-1c).

(4) Place a finger over the tip of the pipet and remove the rubber suction tubing.

(5) Mix the contents of the pipet for about 10 seconds by holding the pipet on a horizontal plane between the thumb and forefinger of one hand and rotating in a "figure-eight." (See figure 5-1d.) A mechanical shaker is used to further mix the contents.

(6) Place a clean hemacytometer coverglass on the counting chamber. Moistening the shoulders on which the coverglass rests aids in keeping the thin coverglass in place.

(7) Mix the specimen in the pipet [as in step (5)] for about 3 minutes to assure even distribution of cells.

(8) Expel the unmixed and relatively cell-free fluid from the capillary portion of the pipet (usually two to three drops).

(9) Place the forefinger over the top (short end) of the pipet, hold the pipet at a 45° angle, and touch the pipet tip to the junction of the coverglass and the counting chamber (see figure 5-2).

(10) Allow the mixture to flow under the coverglass until the chamber is completely charged. Similarly fill the opposite chamber of the hemacytometer. If overflowing into the moat or air bubbles occur, clean and dry the chambers, remix the contents of the pipet, and refill both chambers.

(11) Allow the cells to settle for about 3 minutes. Under low-power magnification, focus on the ruled section of the counting chamber and locate the center one-sq-mm area for counting the red cells.

(12) Observe for even distribution of cells.

(13) Switch to high-power magnification and locate the square in the upper left-hand corner (see figure 5-3).

(14) Count all the red cells lying within this square and those touching the center line of the upper- and right-hand triple lines. The red cells which touch the left-hand and bottom-center lines are not counted.

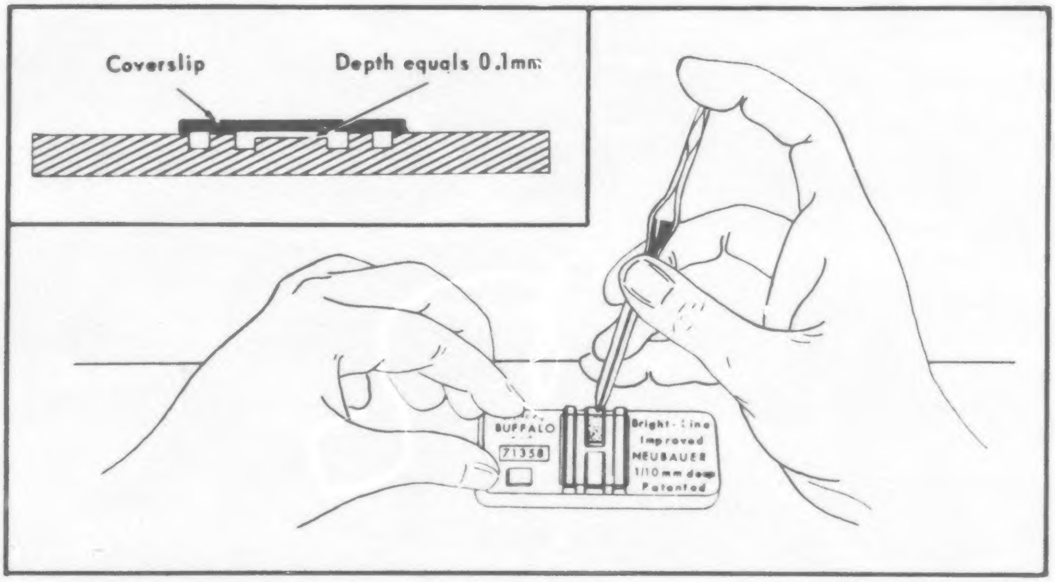


Figure 5-2. Charging the Hemacytometer.

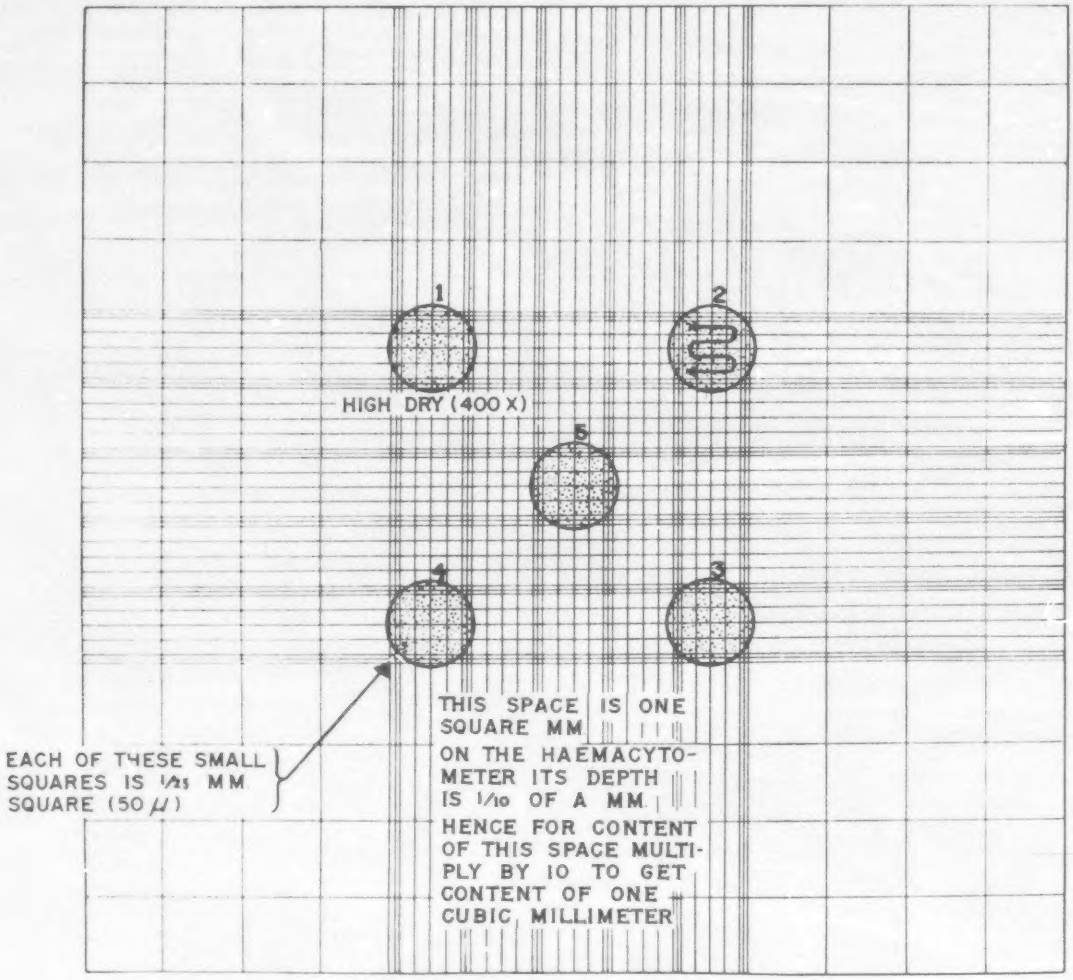

1
2
HIGH DRY (400 X)
5
4
3
THIS SPACE IS ONE SQUARE MM
ON THE HAEMACYTOMETER ITS DEPTH IS 1/10 OF A MM
HENCE FOR CONTENT OF THIS SPACE MULTIPLY BY 10 TO GET CONTENT OF ONE CUBIC MILLIMETER
EACH OF THESE SMALL SQUARES IS 1/25 MM SQUARE (50 µ)

Figure 5-3. Hemacytometer Counting Chamber. Areas marked 1, 2, 3, 4, and 5 are used to count red blood cells.

(15) In the same manner, count the red cells in the remaining four small squares, as illustrated. A variation of more than twenty-five cells between any of the five areas counted indicates uneven distribution. Should this occur, repeat the procedure starting with step (7).

(16) Count the second chamber in the same manner.

d. Calculations:

(1) Routinely, blood is drawn to the 0.5 mark and diluted to the 101 mark with RBC diluting fluid. All the blood is washed into the bulb of the pipet (which has a volume of 100). Therefore, 0.5 volumes of blood are contained in 100 volumes of diluting fluid. The resulting dilution is 1:200. These figures are arbitrary and refer strictly to dilution and not to specific volumetric measurements.

(2) The depth of the counting chamber is 0.1 mm and the area counted is 0.2 sq mm (5 squares are counted each with an area of 0.04 sq mm; therefore, 5 × 0.04 sq mm = a total of 0.2 sq mm). The volume

counted is area × depth = volume or 0.2 sq mm × 0.1 mm = 0.02 cu mm.

(3) The formula is as follows:

Average (of two chambers) number of

$$\text{RBC's counted} \times \frac{\text{dilution (200)}}{\text{volume (0.02)}} = \text{RBCs per cu mm}$$

(4) Example:

First Chamber Cells counted in each square	Second Chamber Cells counted in each square
110	99
100	102
95	87
115	95
90	107
510 RBCs counted	490 RBCs counted

Average of the two chamber counts

$$\begin{array}{r} 510 \\ 490 \\ \hline 1000 \end{array} \qquad \frac{1000}{2} = 500 \text{ RBCs counted}$$

$$\frac{500 \times 200}{0.02} = 5{,}000{,}000 \text{ RBCs per cu mm}$$

e. Sources of Error:

(1) Improper collection of blood specimens causes variable results.

(2) Wet or dirty pipets.

(3) Poor condition or inaccurate calibration of pipets. Pipets must be in good condition and calibrated to have a maximum error of ±1%.

(4) Poor pipetting technique causes high or low counts. Poor pipetting technique includes:

(a) Undershooting desired line with blood or diluting fluid.

(b) Overshooting desired line with blood or diluting fluid.

(c) Air bubbles in the column on bulb.

(d) Failure to wipe tip free of blood.

(e) Too slow manipulation following the withdrawal of the specimen thus allowing some of the blood specimen to coagulate.

(f) Failure to mix the blood and diluent properly.

(5) Failure to expel two to three drops in the pipet tips before charging the hemacytometer.

(6) Overfilling the chamber of the hemacytometer, which causes erroneously-high counts.

(7) Wet or dirty coverglasses and hemacytometers.

(8) Uneven distribution of cells in the counting chamber causes erroneous results.

(9) Inaccuracy or carelessness in making counts.

(10) Diluent which is cloudy or contains debris.

(11) Failure to mix anticoagulated blood thoroughly before use

f. Discussion:

(1) The error resulting from the distribution of cells in the hemacytometer including field and performance errors is ±7 to 11%. Field errors are minimized by counting large numbers of cells. Performance errors are minimized by good technique and equipment.

(2) The minimum blood sample recommended for performing routine red blood cell counts is that obtained using one pipet and counting two chambers as outlined above. However, when the red blood cell count is to be used in the calculation of blood indices, greater accuracy is required. In this case, the procedure is to use two separate pipets and fill two chambers from each pipet. The average count is then obtained and is used in the calculation of the blood indices.

(3) When venous blood is used, it must be mixed properly with an anticoagulant at the time the blood is drawn and again just prior to pipetting to assure uniform distribution of cells. Vigorous mixing should be avoided since hemolysis can result.

(4) If capillary blood is used, the technician must work rapidly following the puncture, otherwise coagulation of the specimen and settling of cells in the specimen are likely to begin before the blood is mixed

with the diluent. This results in a low cell count and/or a clogged pipet. To avoid introducing excess tissue fluids that result from "milking" the finger, do NOT massage the finger after puncture has been made.

(5) It is of the utmost importance that clean, dry diluting pipets be used so that hemolysis is precluded.

(6) Hayem's solution should not be more than 3 weeks old and should be filtered frequently. If the solution stands for any length of time, it becomes cloudy which makes it useless as a red blood cell diluting fluid.

(7) Occasionally, blood "leaks" into the diluting solution from inserted pipets and causes serious errors. Thus, routine filtration is necessary.

(8) Pseudoagglutination of the red cells occurs in cases of abnormal plasma proteins (myeloma, Hodgkin's disease) or with an increase in cold agglutinins (viral pneumonia). This clumping is prevented by adding 0.01 g of gelatin to 100 ml of Hayem's solution. Heating of the Hayem's solution to 64° C counteracts the effect of cold agglutinins without hemolyzing the red blood cells. It is claimed that Gower's solution prevents pseudoagglutination of erythrocytes.

(9) In polycythemia the number of red cells may be too great to permit acccurate enumeration if a dilution of 1:200 is used. Blood is therefore drawn to the 0.3 instead of the 0.5 mark and the sample diluted to the 101 mark in the usual manner. The resulting dilution of 1:333 is used in the calculation.

(10) In anemia the number of red cells may be too small to permit accurate enumeration if a dilution of 1:200 is used. Blood is therefore drawn to the 1.0 instead of the 0.5 mark and the sample diluted to the 101 mark in the usual manner. The resulting dilution of 1:100 is used in the calculation.

g. Normal Values:

(1) Adult Male: 4.2–5.4 million RBCs per cu mm.

(2) Adult Female: 3.6–5.0 million RBCs per cu mm.

(3) Childhood: 3.8–5.4 million RBCs per cu mm.

(4) Birth: 4.7–7.1 million RBCs per cu mm.

5-3. White Blood Cell Count (Hemacytometer):

a. Principle. A sample of blood is diluted with solution which lyses nonnucleated red blood cells. Following adequate mixing, the specimen is introduced into a counting chamber where the white blood cells (leukocytes) in a diluted volume are counted.

b. Reagent. 2% Acetic Acid: Add 2 ml glacial acetic acid to a 100-ml volumetric flask. Dilute to the mark with distilled water. Add 1 drop of 1% gentian violet and mix.

c. Procedure:

(1) Draw well-mixed capillary or venous blood exactly to the 0.5 mark in a white blood cell diluting pipet. This blood column must be free of air bubbles.

(2) Wipe the excess blood from the outside of the pipet to avoid transfer of cells to the diluting fluid. Take care not to touch the tip of the pipet with the gauze.

(3) Immediately draw 2 percent acetic acid diluting fluid to the "11" mark while rotating the pipet between the thumb and forefinger to mix the specimen and diluent. Hold the pipet upright to prevent air bubbles in the bulb. (See figure 5–1.)

(4) Mix the contents of the pipet for 3–5 minutes and fill the counting chamber as described in the technique for red blood cell counts in paragraph 5–1c.

(5) Allow the cells to settle for about 3 minutes. Under low-power magnification and reduced light, focus on the ruled area and observe for even distribution of cells.

(6) Count the white cells in the four 1-sq-mm corner areas corresponding to those marked A, B, C, and D of figure 5–4 in each of two chambers.

(7) Count all the white cells lying

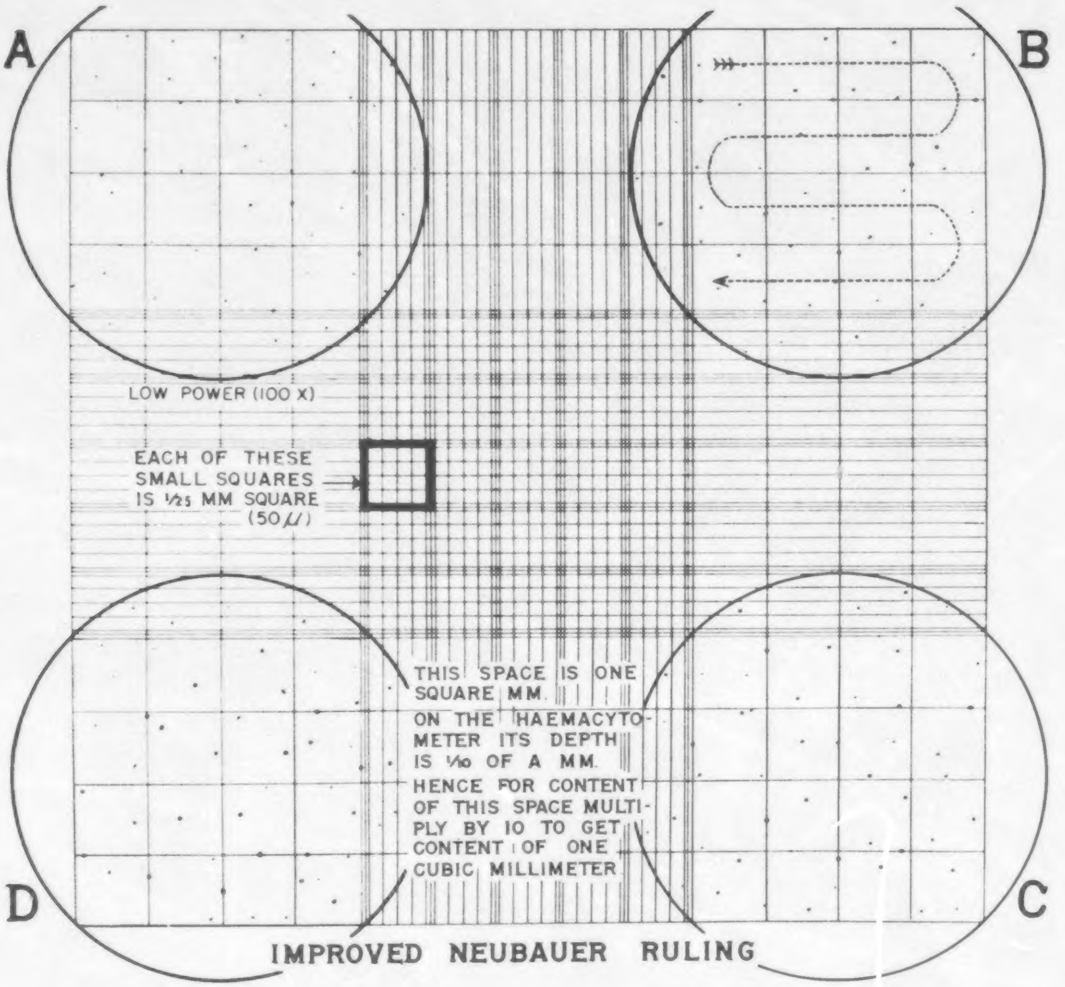

A
B
LOW POWER (100 X)
EACH OF THESE SMALL SQUARES IS 1/25 MM SQUARE (50μ)
THIS SPACE IS ONE SQUARE MM.
ON THE HAEMACYTOMETER ITS DEPTH IS 1/10 OF A MM.
HENCE FOR CONTENT OF THIS SPACE MULTIPLY BY 10 TO GET CONTENT OF ONE CUBIC MILLIMETER
D
C
IMPROVED NEUBAUER RULING

Figure 5-4. Hemacytometer Counting Chamber. Areas marked A, B, C, and D are used to count white blood cells.

within the square and those touching the upper and right-hand center lines. The white cells which touch the left-hand and bottom lines are not to be counted. In each of the four areas conduct the count as indicated by the "snake-like" line in the figure 5-4. A variation of more than 10 cells between any of the four areas counted indicates uneven distribution and requires that the procedure be repeated.

d. Calculation:

(1) Routinely, blood is drawn to the 0.5 mark and diluted to the 11 mark with WBC diluting fluid. All the blood is washed into the bulb of the pipet (which has a volume of 10). Therefore, 0.5 volumes of blood are contained in 10 volumes of diluting fluid. The resulting dilution is 1:20. These figures are arbitrary and refer strictly to dilution and not to specific volumetric measurements.

(2) The depth of the counting chamber is 0.1 mm and the area counted is 4 sq mm (4 squares are counted, each with an area

of 1.0 sq mm; therefore, 4 × 1.0 sq mm = a total of 4 sq mm). The volume counted is: area × depth = volume. Four sq mm × 0.1 mm = 0.4 cu mm.

(3) The formula is as follows:

Average (of two chambers) number of

$$\text{WBCs counted} \times \frac{\text{dilution (20)}}{\text{volume (0.4)}} = \text{WBCs per cu mm}$$

(4) Example:

First Chamber Cells counted in each square	Second Chamber Cells counted in each square
35	45
40	37
44	36
39	44
158 WBCs counted	162 WBCs counted

Average of the two chamber counts:

$$\begin{array}{r} 158 \\ 162 \\ \hline 320 \end{array} \qquad \frac{320}{2} = 160 \text{ WBCs}$$

$$\frac{160 \times 20}{0.4} = 8{,}000 \text{ WBCs per cu mm}$$

e. Sources of Error. See paragraph 5-1c.

f. Discussion:

(1) The allowable error when four large squares are counted is ±20%. Counting eight large squares decreases the error to ±15%.

(2) The importance of clean, dry diluting pipets cannot be stressed too much as the greatest source of error in the counting of both white and red blood cells is the use of wet and/or dirty pipets.

(3) The counting chamber must be scrupulously clean and free of debris which might be mistaken for cells.

(4) The minimum blood sample recommended for performing routine white blood cell counts is that obtained using one pipet and counting two chambers as previously outlined.

(5) In cases where the white cell count is exceptionally high, as in leukemia, the dilution should be made in the red blood cell diluting pipet. The blood is drawn to the 1.0 mark and the diluting fluid (2 percent acetic acid) is drawn to the 101 mark. The resulting dilution is 1:100.

(6) In cases of leukopenia, the white pipet should be filled to the 1.0 mark and diluted to the 11 mark with 2 percent acetic acid. The resulting dilution is 1:10.

(7) If nucleated erythrocytes are present, the count is corrected by the folllowing formula:

$$\text{observed count} \times \frac{100}{100 + \%\ \text{nucleated erythrocytes}}$$

The percent nucleated erythrocytes is obtained from the differential count (see paragraph 5-2b).

(8) Unopette procedures are available for RBC and WBC counts.

g. Normal Values:

(1) Adults (both sexes): 4,500-11,500 WBCs per cu mm.

(2) Childhood: 6,000-14,000 WBCs per cu mm.

(3) Birth: 9,000-30,000 WBCs per cu mm.

5-4. Total Eosinophil Count:

a. Principle. A sample of blood is diluted with a solution which selectively stains the eosinophils and eliminates all other leukocytes and erythrocytes from view. Following mixing, the specimen is introduced into the counting chamber and the number of eosinophils in a known volume of blood is counted.

b. Reagent. Pilot's Solution: Add 50 ml propylene glycol, 40 ml distilled water, and 1.0 ml sodium carbonate (10% aqueous) to a 150-ml beaker. Mix well and filter. Add 10 ml of phloxine (1% aqueous) just prior to use. The addition of 10 units of heparin to this solution prevents clumping of cells.

c. Procedure:

(1) Draw capillary (or venous blood) to the 1.0 mark in each of two white cell diluting pipets.

(2) Draw Pilot's solution to the 11 mark of each pipet.

(3) Mix by gently shaking the pipets for 30 seconds. Prolonged and harsh shaking will tend to cause rupturing of the eosinophils.

(4) Expel the cell-free liquid from the capillary portion of the pipets.

(5) Using one pipet, charge both chambers of a hemacytometer and with the other pipet charge both chambers of the second hemacytometer.

(6) Allow both hemacytometers to stand for 15 minutes to permit staining of the eosinophils. To prevent evaporation, the hemacytometers are placed on a damp towel and covered with Petri dish covers.

(7) Under low-power magnification, count the red-stained eosinophils in the entire ruled area (9 sq mm) on each of the four chambers (a total area of 36 sq mm). The chamber has a depth of 0.1 mm so the total volume is 3.6 cu mm.

d. Calculations:

$$\text{Number of eosinophils counted} \times \frac{\text{dilution (10)}}{\text{volume (3.6)}} = \text{eosinophils per cu mm}$$

e. Sources of Error. See paragraph 5-2e.

f. Discussion:

(1) Fuchs-Rosenthal or Levy hemacytometer is preferable to a standard counting chamber since its greater volume (4.0 x 4.0 x 0.2 mm) allows for counting of more cells, thereby reducing the statistical error. Counting two of these chambers is equivalent in accuracy to seven standard chamber counts.

(2) In eosinopenia, it is necessary to set up more chambers to provide an optimum number of cells to be counted.

(3) The eosin-acetone diluting fluids are unsatisfactory and should not be used.

(4) Estimation of eosinophils on a stained blood smear are too inaccurate for use because of poor cellular distribution.

(5) The propylene glycol in Pilot's solution renders the erythrocytes invisible, and the sodium carbonate causes lysis of all the leukocytes except the eosinophils. The phloxine stains the eosinophils.

(6) In the Thorn test an eosinophil count must be made prior to the initiation of the test proper. This establishes the patient's total eosinophil count, to which the response of the adrenal cortex to ACTH can be judged. ACTH is then injected and at an interval of 4 hours, another eosinophil count is made. The interpretation of this test is as follows:

Normal—approximately a 50 percent drop in eosinophils.
Cushing's disease (hyperadrenalism)—0-30 eosinophils per cu mm.
Addison's disease (hypoadrenalism) no change in eosinophil count.

(7) Nasal smears are also submitted for eosinophil evaluation. These smears are stained with Wright's stain and examined for the presence of eosinophils.

g. Normal Value: 150-300 eosinophils per cu mm.

5-5. Absolute Basophil Count (Cooper):

a. Principle. Erythrocytes are lysed with cetylpyridinium chloride. The basophils are rendered insoluble in ammonium sulfate and stained with toluidine blue. The stained basophils are then counted in a Fuchs-Rosenthal counting chamber.

b. Reagents:

(1) Solution 1. Add 100 mg of EDTA (disodium salt) to a 100-ml volumetric flask. Dilute to the mark with 0.85% NaCl.

(2) Solution 2. Mix 25 ml of 0.5% cetylpyridinium chloride solution (aqueous), 20 ml of 0.8% toluidine blue in 5% aluminum sulfate, and 20 ml of distilled water in a beaker. Filter and transfer to a reagent bottle.

c. Procedure:

(1) Add 0.08 ml solution 1 to a 10 x 75 mm test tube.

(2) Add 0.02 ml blood from a skin puncture and mix.

(3) Add 0.1 ml solution 2; mix.

(4) Fill 2 Fuchs-Rosenthal chambers using a capillary pipet.

(5) Place the chambers in a moist atmosphere and allow the cells to settle for 5 minutes.

(6) Count the basophils in all sixteen large squares. The basophils are purple-red, metachromatically-stained cells.

d. Calculations. The chamber has an area of 16 sq mm and a depth of 0.2 mm; therefore, the volume count equals 16 x 0.2 or 3.2 cu mm. The formula is as follows:

$$\frac{\text{Total basophils counted}}{3.2} = \text{Basophils per-cu mm}$$

e. Sources of Error. See paragraph 5-2e.

f. Discussion:

(1) An absolute basophil count is used to study allergic reactions.

(2) The EDTA solution prevents platelet agglutination.

(3) The aluminum sulfate acts as a mordant to improve the staining qualities of toluidine blue.

(4) Another procedure employed to perform a basophil count is the Neutral Red Technique. It has the following disadvantage: Eosinophils can be stained, basophils can be water soluble, and platelet aggregates can form.

(5) Basopenia refers to a decreased number of basophils. Basophilia refers to an increased number of basophils.

5-6. Reticulocyte Count:

a. Principle. Nonnucleated immature erythrocytes (diffusely basophilic erythrocytes) retain some basophilic substance. With supravital staining this substance appears as a reticulum within the cell. This cell is then known as a reticulocyte.

b. Reagent. New Methylene Blue Solution. Dissolve 0.5 grams of new methylene blue, 1.4 grams potassium oxalate, and 0.8 g NaCl in distilled water. Dilute to 100 ml. Filter before use.

c. Procedure:

(1) Add 3 or 4 drops of new methylene blue and 3 or 4 drops of blood (venous or capillary) to a small test tube or mix in a microhematocrit tube. Mix well and let stand for 15 minutes.

(2) Place a small drop on a clean glass slide and prepare a thin smear (see chapter 3, paragraph 3-6).

(3) After the slide has dried, focus on the smear under low-power magnification and locate the thin portion of the smear.

(4) Switch to oil immersion magnification and count 200 erythrocytes, including reticulocytes in each of five different areas on the smear (a total of 1,000 cells). Record the number of reticulocytes observed.

d. Calculation:

$$\frac{\text{Number of reticulocytes counted}}{10} = \%\ \text{reticulocytes}$$

e. Sources of Error:

(1) Equal volumes of blood and stain give optimum staining conditions. An excess of blood causes the reticulum to understain. An excess of stain usually obscures the reticulum.

(2) Crenated erythrocytes and rouleaux formation make an accurate count difficult to perform.

(3) Stain precipitated on erythrocytes causes them to appear as reticulocytes.

(4) Dirty slides causes uneven spreading.

(5) The dye solution should have adequate time (step 1) to penetrate the cell and stain the reticulum.

f. Discussion:

(1) Reticulocytes are nonnucleated erythrocytes which exhibit blue reticulum strands within their cytoplasm when stained supravitally. When stained only with Wright's stain, they are buff-pink in color and larger and darker than erythrocytes.

(2) Reticulocytes serve as an index of the activity of the bone marrow in blood regeneration. As such, these counts are of value in following anti-anemia therapy. Satisfactory response to therapy is evidenced by an increase of reticulocytes in the peripheral blood. Increased reticulocyte counts also occur whenever there is rapid bone marrow activity as in leukemia or blood regeneration associated with hemorrhage or hemolysis.

(3) Several methods for staining and counting reticulocytes are in common use. Compared to the use of alcoholic solutions of dye, methods employing saline solutions of new methylene blue can give slightly higher values for reticulocytes. For comparative studies, the same method should be used throughout the work.

(4) Precipitated stain is often confused with reticulum but can be recognized by its presence throughout the smear and apart from the red cells. Precipitation can be eliminated as a source of error by frequently filtering the stain.

g. Normal Values:

(1) Birth: 2.5–6.0%.

(2) Adults (both sexes): 0.5–1.5%.

5–7. Cerebrospinal Fluid Cell Counts:

a. Principle. Cerebrospinal fluid is delivered to a counting chamber and examined microscopically for blood cells. Normally, spinal fluid is clear. If the spinal fluid is cloudy, a dilution is made before charging the counting chamber.

b. Reagent. Cerebrospinal Fluid Diluting Fluid: Add 10 ml of glacial acetic acid and 0.2 grams of crystal violet to a 100–ml volumetric flask. Dilute to the mark with distilled water.

c. Procedure:

NOTE: Set up cell counts on spinal fluids within 30 minutes after withdrawal of the specimen.

(1) Clear spinal fluid is set up as follows:

(a) With a capillary pipet introduce a drop of well-mixed spinal fluid into one counting chamber of a hemacytometer. (CAUTION: Avoid contamination by careful handling of spinal fluid.)

(b) Examine the entire ruled area for the presence of cellular elements. If both leukocytes and erythrocytes are observed, note the condition of the red cells (fresh or crenated).

(c) Count all cells in the entire ruled area (0.9 cu mm).

(2) Turbid spinal fluid is set up as follows:

(a) Draw cerebrospinal fluid diluting fluid to 1.0 mark of the white blood cell diluting pipet.

(b) Carefully draw a well-mixed specimen of spinal fluid to the 11 mark.

(c) Shake the pipet for 2 minutes to mix the specimen.

(d) Discard the fluid in the capillary portion of the pipet.

(e) Charge the counting chamber and allow the cells to settle for 5 minutes.

(f) Under low-power magnification count all cells in the entire ruled area (0.9 cu mm).

(g) Switch to high-power and perform a rough differential count.

(3) For very cloudy spinal fluid a white blood cell dilution is made as follows:

(a) Draw spinal fluid to the 0.5 mark in the white blood cell diluting pipet.

(b) Draw cerebrospinal fluid diluting fluid to the 11 mark.

(c) Perform steps (c) through (g) in paragraph 5–6c(2).

d. Calculations:

(1) Clear spinal fluid:

$$\frac{\text{Number of cells counted}}{\text{volume (0.9)}} = \text{cells per cu mm}$$

(2) Turbid spinal fluid:

$$\frac{\text{Number of cells counted} \times \text{dilution (10/9)}}{\text{volume (0.9 cu mm)}} = \text{cells per cu mm}$$

(3) Very clouded spinal fluid:

$$\frac{\text{Number of cells counted} \times \text{dilution (20)}}{\text{volume (0.9 cu mm)}} = \text{cells per cu mm}$$

e. Sources of Error. See paragraph 5-2e.

f. Discussion:

(1) If more than 100 leukocytes per cu mm are present, centrifuge the undiluted specimen, make a smear, and stain with modified Wright's stain. Perform a routine differential count and also estimate the ratio of erythrocytes to leukocytes. NOTE: It may be necessary to use egg albumin or cell-free serum to make the sediment adhere to the slide.

(2) Normally the spinal fluid is water-clear. It can be turbid if cell count is 500 or more cells per cu mm. If there is frank blood with spontaneous clotting, the indications are those of a bloody tap. Xanthochromia develops after subarachnoid hemorrhage has been present for a few hours and is due to disintegration of blood pigments. Xanthochromia may also develop from tumors, abscesses, and inflammation.

(3) Cell counts above 10 are considered to be evidence of intracranial disease. The predominant cell in most viral infections, syphilis, and tuberculous meningitis is the lymphocyte. Bacterial infections due to meningococcus, pneumococcus, etc., usually result in a predominance of the neutrophil. Cerebral and extradural abscesses as well as subdural hemorrhages produce a neutrophilic response although bacteria are not demonstrated.

(4) Biochemical, bacteriological, virological, serological, and hematological examinations are all necessary to reflect the true condition of the cerebrospinal fluid. The current laboratory standing operating procedures should give guidance to the efficient method to accomplish all the necessary examinations.

g. Normal Value: 0–5 cells per cu mm (chiefly lymphocytes).

5-8. Semen Analysis:

a. Principle. Semen analysis involves gross examination (volume, color, turbidity, viscosity, and pH) and microscopic examination (motility and spermatozoa count).

b. Reagent. Spermatozoa Fixative Solution: Add 5 grams sodium bicarbonate ($NaHCO_3$) and 1 ml of formalin to a 100-ml volumetric flask. Dilute to the mark with distilled water.

c. Collection Instruction. A physician will usually give the instruction; however, the patient should be reminded of several critical points.

(1) The patient may be required to abstain from intercourse for a period directed by the physician.

(2) The specimen is collected in a clean container that has been prewarmed to body temperature.

(3) The specimen should be delivered to the laboratory within 30 minutes.

(4) The specimen must be kept at body temperature (37° C) and not subjected to extremes of heat or cold.

d. Gross Examination:

(1) Record the time of collection and receipt of the specimen.

(2) Measure and record the volume.

(3) Observe and record the color (white, gray, yellow, etc.), turbidity (clear, opalescent, opaque, etc.), and viscosity (viscid, gelatin, liquid).

(4) Determine the pH with a pH reagent strip and record this.

e. Motility Examiniation:

(1) When the specimen becomes fluid (within 15 to 30 minutes after collection the semen liquefies by the action of fibrinolysin), place 1 drop on a slide (prewarmed to 37° C) and place a coverslip on it.

(2) Under high dry power, count motile and nonmotile spermatozoa in two or more areas. Only those which move forward

actively are considered motile. Record the percent of motile spermatozoa seen.

(3) Repeat this procedure in 3 hours and 6 hours, using a new drop from the original specimen each time.

f. Spermatozoa Count:

(1) Draw the liquefied semen to the 0.5 mark on white blood cell diluting pipet.

(2) Draw fixative solution to the 11 mark on the WBC pipet.

(3) Let the mixture stand until the mucus dissolves.

(4) Shake the pipet thoroughly and charge a hemacytometer.

(5) Count the spermatozoa in the same manner as you would count white blood cells.

(6) After counting the sperm, examine the morphology and report the percent of abnormal forms. Morphologically-normal sperm are quite uniform in appearance. Any sperm with rounded, enlarged, small, or bilobed heads are abnormal. Abnormal tails are enlarged, small, irregular in length, absent, or multiple. See figure 5-5 for morphology of spermatozoa.

g. Calculations:

(1)
$$\frac{\text{Number of sperm counted} \times \text{dilution (20)}}{\text{volume (0.4)}} = \text{sperm per cu mm}$$

(2) Sperm per cu mm x 1000 = sperm per ml

h. Sources of Error:

(1) Delay in analysis results a lower percentage of motile forms and a lower count.

(2) Temperature extremes cause spermatozoa to die.

(3) See paragraph 5-2e for sources of error when counting.

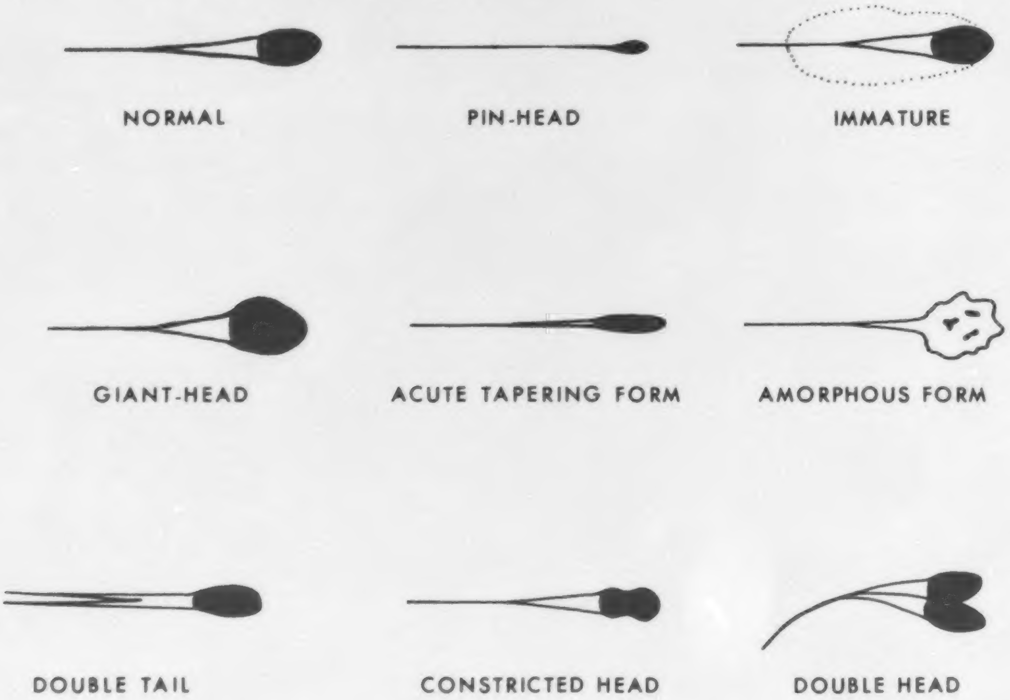


Figure 5-5. Morphology of Spermatozoa.

i. Discussion:

(1) Semen analyses are usually performed for sterility studies following a vasectomy or a barren marriage.

(2) Semen analysis can be performed for medico-legal cases involving rape or to support or disprove a denial of paternity on the grounds of sterility.

(3) Semen is derived from the following: testes, seminal vesicles, prostate, epididymides, vasa deferentia, bulbo-urethral glands, and urethral glands.

j. Normal Values:

(1) Volume: 1.5–5.0 ml.

(2) pH: 7.2–7.6.

(3) Motility: 60–90%.

(4) Spermatozoa Count: 60–150 million per ml.

SECTION B—HEMATOCRIT

5-9. Introduction:

a. The hematocrit or the packed-cell volume is the percentage of the total volume of red blood cells in relation to the total volume of whole blood. The procedure is performed by filling a tube (capillary or Wintrobe) with blood and centrifuging at constant speed for a constant period of time. The packed-cell volume is then measured. The hematocrit can also be determined by automated sequential analyzers but is usually a calculated value. Two manual methods are presented in this chapter: the macromethod of Wintrobe and a micromethod.

b. The hematocrit is the most useful single index of the degree of anemia or polycythemia. It can be the most accurate (2–4 percent error) of all hematological determinations. In contrast, the direct red blood cell chamber count has a percent error of 8–10%. The hematocrit is, therefore, preferable to the red blood cell count as a screening test for anemia. Erythrocyte counts and hemoglobin can be estimated from the hematocrit by the following formulas:

(1) One hematocrit point = 0.34 gram hemoglobin per 100 ml of blood.

(2) One hematocrit point = 107,000 erythrocytes per cubic millimeter of blood.

5-10. Macrohematocrit (Wintrobe):

a. Principle. Whole blood is collected by venipuncture, mixed with an anticoagulant, and pipetted into a Wintrobe tube. The tube is then centrifuged to pack the cellular elements. The height of the packed red cell column is read and reported as a percentage of the total blood column. This percent is the hematocrit or packed-cell volume (PCV).

b. Procedure:

(1) Draw 5 ml of blood by venipuncture, place the blood in a test tube containing EDTA, and mix thoroughly by gently inverting the tube several times.

(2) Draw the blood into a capillary pipet. Fill the Wintrobe tube to the "0" mark by inserting the pipet to the bottom of the tube, while holding the tube at a 45° angle. As the tube is filled, slowly withdraw the pipet so that the tip is always just below the level of the blood. The blood column must be free of bubbles.

(3) Place the Wintrobe tube in a centrifuge and spin at 3,000 rpm for 30 minutes. The RCF must be 2250 G.

(4) Read the height of the column of packed red cells at its junction with the buffy coat. This level, as shown on the ascending scale etched on the tube, is the hematocrit and is reported in percentage (see figure 5-6).

c. Sources of Error:

(1) Failure to mix the blood specimen thoroughly yields an unrepresentative sample. Always remix the sample immediately prior to filling the tube.

(2) Prolonged application of the tourniquet causes a concentration of blood cells. This results in an elevated value.

(3) The sample must be spun for 30 minutes at 3,000 rpm. Neither the time nor

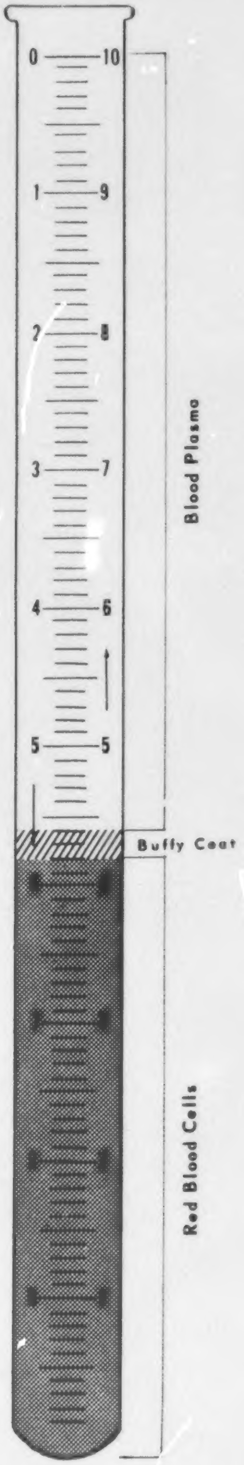


Figure 5-6. Wintrobe Hematocrit Tube.

the speed can be varied without altering the results. The RCF must be 2250 G.

(4) Misreading the red cell level by including the buffy coat yields an increased result.

d. Discussion:

(1) To allow for better separation of the cellular elements in the hematocrit, the tube should stand for at least 15 minutes prior to centrifugation. This separation is adequately accomplished if a 1-hour sedimentation rate precedes the hematocrit.

(2) Above the column of packed red cells is a layer of white cells and thrombocytes called the "buffy coat." This layer is normally about 0.5 to 1.0 mm in thickness and serves as a rough guide of white cell and platelet numbers. It should not be substituted for the white cell count as variations in size of cells influence the number estimated.

(3) The appearance of the plasma might suggest certain abnormalities. Normally, this fluid is clear and straw colored. A golden-yellow colored plasma might indicate the presence of jaundice while a creamy-opaque appearance would suggest an excess of fat in the blood (lipemia).

(4) In normal blood centrifuged at 3,000 rpm for 30 minutes, trapped plasma comprises an average of 4.3 percent of the red cell column. This is reduced to 2.5 percent by centrifuging for 55 minutes. Anisocytosis and spherocytosis do not alter plasma trapping. Variation in the amount of plasma trapping is so small after centrifuging for 55 minutes, even with pathological bloods, that it is recommended over the 30-minute centrifugation when greater accuracy is desired.

e. Normal Values:

(1) Birth: 44–64% (average 54%).

(2) Childhood: 34–41% (average 38%).

(3) Adult Males: 40–54% (average 47%).

(4) Adult Females: 38–47% (average 42%).

5-11. Microhematocrit:

a. Principle. A capillary tube is filled with whole blood by gravity and capillarity to within 1 or 2 cm of the end. The unfilled end is sealed and the tube is centrifuged. After centrifugation, the capillary tube is placed in a reading device and the hematocrit value determined.

b. Procedure:

(1) If anticoagulated venous blood is the specimen, fill a plain capillary tube with blood. If blood without anticoagulant is used, fill a heparinized capillary tube with the blood specimen. A heparinized capillary tube is identified by a red line on the tube.

(2) Seal the unfilled end of the tube with a commercial sealing wax or clay.

(3) Place the capillary tube in one of the numbered slots in the centrifuge head in such a manner that the sealed end is in contact with the peripheral rim of the centrifuge head. CAUTION: Failure to have the capillary tube in contact with the rim might result in a shattered tube as the centrifuge speeds up.

(4) Screw the flat centrifuge head cover in place.

(5) Centrifuge the capillary tube at 10,000 rpm for 5 minutes.

(6) Determine the hematocrit value with the aid of a microhematocrit reader. NOTE: Since there are a variety of readers available, it is necessary that the technician carefully follow the directions of the manufacturer for the particular device utilized.

c. Sources of Error:

(1) Improper sealing of the capillary tube causes the blood to blow out of the capillary tube during centrifugation.

(2) Capillary tubes must be properly identified. Numbered holders for capillary tubes are available. Place a tube in a slot on the holder and record the number on the laboratory request slip.

(3) Improper centrifugation leads to varied results. For good quality control, maintain prescribed centrifuge speed and time.

(4) Misreading the red cell level by including the buffy coat causes elevated values.

d. Discussion:

(1) The microhematocrit technique is advantageous because of speed, and because only a small quantity of blood is necessary for the determination. An additional advantage is the ease with which this procedure is adapted to infants and small children. The microhematocrit technique requires only a simple capillary puncture whereas in the Wintrobe method venous blood must be used. Another advantage is the use of disposable capillary tubes.

(2) If the microhematocrits cannot be read promptly, the capillary tubes must be properly identified and placed in a vertical position. Slanting of the cell layer will occur if tubes are left in a horizontal position for more than 30 minutes.

e. Normal Values:

(1) Birth: 44–64% (average 54%).

(2) Childhood: 34–41% (average 38%).

(3) Adult Males: 40–54% (average 47%).

(4) Adult Females: 38–47% (average 42%).

SECTION C—ERYTHROCYTE SEDIMENTATION RATE

5-12. Introduction. The erythrocyte sedimentation rate (ESR) measures the rate of settling of erythrocytes out of the cellular-plasma suspension. The ESR is complex biological phenomenon influenced by a variety of factors. Size and shape of erythrocytes cause the ESR to fluctuate. Microcytes tend to settle slower than normal cells, while macrocytes fall more rapidly. An increase in spherocytes and/or bizarrely-shaped red cells retards the sedimentation rate. With a decreased hematocrit there is less retardation of sedimentation by the erythrocytes themselves and they tend to settle faster. Corrections for anemic blood are available; however, most experts consider this correction useless and invalid. Always correct for anemia when requested to do so by a physician. An increased hematocrit (above 55%) retards the sedimentation rate. In certain disease processes there is an increase in fibrinogen and/or globulin. The increased concentration of these substances causes the erythrocytes to settle faster. The determination of the erythrocyte sedimentation rate is performed by many methods. The most popular, the Wintrobe-Landsberg, is outlined below.

5-13. Determination of Sedimentation Rate (Wintrobe-Landsberg):

a. Principle. Anticoagulated blood is placed in a narrow tube. The blood cells settle out of the suspension, leaving clear plasma above them. The distance that the erythrocytes fall within a given interval of time is measured.

b. Procedure:

(1) Draw 5 ml of blood by venipuncture and place in a test tube containing EDTA (lavender top vacuum tube).

(2) Thoroughly mix the blood and anticoagulant by gently inverting the tube several times, being careful not to cause bubbles.

(3) Draw the blood into the capillary pipet and fill the Wintrobe tube to the "0" mark. This is done by inserting a capillary pipet to the bottom of the Wintrobe tube, while holding it at an angle of 45°. As the tube is filled, slowly withdraw the pipet so that the tip is always just below the level of the blood. The blood column must be free of bubbles.

(4) Place the filled Wintrobe tube in a rack in an exactly vertical position and note the time and room temperature.

(5) At the end of exactly 1 hour, read the level to which the red cells have settled on the descending scale etched on the tube. Each mark equals 1.0 mm while each numbered mark equals 10 mm (1 cm). The figure obtained is reported in mm per hour as the "uncorrected" erythrocyte sedimentation rate.

(6) If a "corrected" sedimentation is requested, perform a hematocrit. Calculators to correct for sedimentation rate are available in the Federal Supply Catalog.

c. Sources of Error:

(1) The blood specimen must be properly mixed with the proper anticoagulant to obtain an undiluted representative sample.

(2) Delay in performing the test beyond 2 hours after the blood is drawn decreases the sedimentation rate.

(3) Increase in temperature accelerates the rate. Desirable temperature range is 22° C to 27° C.

(4) The tube must be vertical. A 3° variation from the vertical accelerates the rate by 30%.

(5) Dirty Wintrobe tubes or capillary pipets can decrease the rate.

(6) Tubes should be placed free from vibration or disturbance.

d. Discussion:

(1) The erythrocyte sedimentation rate is a nonspecific test which suggests the possibility of a disease process and tissue damage in the body. It is not diagnostic but is extremely useful in following the course of some diseases.

(2) The rate is usually increased in inflammatory infections, toxemia, cell or tissue destruction, severe anemia, active tuberculosis, syphilis, acute coronary thrombosis, rheumatoid arthritis, and malignant processes.

(3) Sickle cell anemia, polycythemia, hypofibrinogenemia and certain drugs usually decrease the rate.

e. Normal Values:

(1) Men: 0-9 mm.

(2) Females: 0-20 mm.

(3) Children: 0-13 mm.

SECTION D—HEMOGLOBIN

5-14. General Information. Hemoglobin is a conjugated protein composed of the basic protein globin linked to 4 heme molecules. Ninety-eight percent of all the iron found in the blood is contained in hemoglobin. Hemoglobin transports oxygen and carbon dioxide. This important substance reacts with oxygen to form oxyhemoglobin. In the tissues oxygen is released and reduced hemoglobin formed. Hemoglobin can react with acids, bases, and oxidizing and reducing agents. It also can exist in a variety of forms. These hemoglobin compounds and variants are discussed briefly in the following paragraphs. For more detailed information, refer to the standard hematological texts.

5-15. Compounds of Hemoglobin:

a. Oxyhemoglobin. Oxygen combines loosely with the iron (ferrous state) in hemoglobin. The loosely attached oxygen diffuses into the tissues for oxidative processes. The hemoglobin then binds carbon dioxide and exists as reduced hemoglobin.

b. Carboxyhemoglobin. Hemoglobin combines with carbon monoxide to form carboxyhemoglobin. Carbon monoxide has an affinity 200 times greater for hemoglobin than oxygen does. Hemoglobin in this combination is incapable of oxygen transport.

c. Methemoglobin. This compound is formed when the ferrous state of the heme is oxidized to the ferric state. This compound is incapable of oxygen transport.

d. Sulfhemoglobin. This compound results from the combination of inorganic sulfides and hemoglobin. This compound is incapable of oxygen transport.

e. Cyanmethemoglobin. This compound results when methemoglobin combines with the cyanide radical. This compound is used in hemoglobinometry.

5-16. Variations of Hemoglobin. The variations of hemoglobin occur due to structural differences in the globin protein. These differences are genetically controlled. The normal hemoglobin components are hemoglobin A (HbA), hemoglobin A_2 (HbA_2), and fetal hemoglobin (HbF). HbA constitutes most of the hemoglobin of a normal adult while HbA_2 constitutes a much smaller amount. HbF is present during the first 4 to 6 months of life and not normally present in adults. Hemoglobin S and hemoglobin C are the most commonly occurring abnormal hemoglobins. Others (D, E, H, etc.) are found in rare occurrence associated with several types of anemia. The various types of hemoglobin are separated by electrophoresis.

5-17. Hemoglobinometry. The hemoglobin concentration is directly proportional to the oxygen-combining capacity of blood. Therefore, the measurement of the hemoglobin concentration in the blood is important as a screening test for diseases associated with anemia and for following the response of these diseases to treatment. There are four basic ways to measure the hemoglobin concentration: (1) measurement of the oxygen-combining capacity of blood (gasometric), (2) measurement of the iron content (chemical method), (3) colorimetric measurement of specific gravity (gravimetric method), and (4) colorimetric measurement of a colored derivative of hemoglobin. The fourth method is the most widely used. The cyanmethemoglobin method is the method of choice and is recommended by the Technical Subcommittee on Haemoglobinometry of the International Committee, for Standardization in Haematology.

5-18. Cyanmethemoglobin Method:

a. Principle. Blood is diluted with a dilute solution of potassium ferricyanide and potassium cyanide at a slightly alkaline pH. The ferricyanide converts the hemoglobin to methemoglobin. The cyanide then reacts with the methemoglobin to form the stable cyanmethemoglobin. The hemoglobin content is then determined in a spectrophotometer.

b. Reagents:

(1) Drabkin's Reagent (Cyanmethemoglobin Reagent). Available in pellets or powder in the Federal Supply Catalog.

(2) Cyanmethemoglobin Standards. Stable, accurate standards of cyanmethemoglobin are available commercially. These solutions usually contain about 80 mg per dl hemoglobin as cyanmethemoglobin. For example, if a 1:301 (0.02 ml to 6.0 ml) dilution is used in the procedure, an 80 mg per dl standard corresponds to: 80 × 301/1000 = 24 g hemoglobin per dl in undiluted blood. Standards of other concentrations and dilutions may be calculated by this same formula.

c. Example of Preparation of a Calibration Curve:

(1) With an 80 mg per dl standard, set up standards as follows:

Tube	*Standard*	*Cyanmethemoglobin Diluent*	*Concentration*
1	0 ml	6 ml	0
2	1.5 ml	4.5 ml	6 g per dl
3	3 ml	3 ml	12 g per dl
4	4.5 ml	1.5 ml	18 g per dl
5	6 ml	0 ml	24 g per dl

(2) Place tube 1 in the well of the spectrophotometer at a wavelength of 540 nm. Adjust the spectrophotometer to zero absorbance.

(3) Read and record absorbance for tubes 2-5.

(4) Plot the absorbance versus hemoglobin concentration on linear graph paper.

(5) Check the calibration daily with one of the prepared standards. NOTE: The hemoglobin standards should be capped and stored in the refrigerator until used. When used to calibrate for hemoglobin determinations, they are allowed to warm up to room temperature to minimize condensation of moisture from the air on the cuvets.

d. Procedure:

(1) Pipet (using suction bulb) exactly 6 ml of Drabkin's solution into a cuvet.

(2) Draw well-mixed venous or capillary blood exactly to the 0.02 ml mark of a calibrated Sahli pipet. Use of a properly calibrated auto-dilutor is recommended.

(3) Wipe the excess blood from the outside of the pipet and add the contents of the pipet to the cuvet containing the Drabkin's solution.

(4) Rinse the pipet several times with the contents of the cuvet to assume complete delivery of the blood sample. Mix well and allow to stand at room temperature for 10 minutes.

(5) At a wavelength of 540 nm, set the spectrophotometer to zero absorbance with the blank cuvet. The blank contains 6 ml of fresh Drabkin's solution.

(6) Read and record the absorbance of the unknown.

e. Calculations. Obtain the grams hemoglobin per dl from the calibration curve.

f. Sources of Error:

(1) Inaccurate calibration of Sahli pipets results in errors in accuracy and precision.

(2) The spectrophotometer must be standardized accurately to obtain valid results.

(3) Cuvets that are dirty, wet, scratched, or mismatched are sources of error.

(4) Lipemic blood produces turbidity in solution which can elevate results.

(5) Heavy smokers usually have results 10 percent lower because of the presence of carboxyhemoglobin. Carboxyhemoglobin requires a full hour to be converted to cyanmethemoglobin.

(6) Drabkin's solution deteriorates rapidly in light. It should be stored in a dark brown bottle and refrigerated.

g. Discussion:

(1) Precautions in the Use of Cyanide:

(a) Always fill pipets with suction bulb, and never by application of suction by mouth. Use automatic dilutors to dispense Drabkin's solution whenever possible.

(b) Immediately clean up any spilled Drabkin's solution with a wet cloth. Dispose of the solution with a wet cloth. Dispose of the solution by flushing it down the sink with water.

(c) Store Drabkin's reagent pellets or powder in a locked cabinet.

(d) Exercise extreme care when preparing Drabkin's solution. This solution is poisonous. Take precautions to avoid getting the solution into the mouth and inhaling the fumes.

(e) Antidotes for cyanide with outlined procedures for administration should be posted in a conspicuous place in the laboratory. Speed in giving the antidote treatment is essential. Below is a suggested procedure which may be adopted:

- Summon a doctor, but start treatment immediately!
- Give amyl nitrite by inhalation (break capsule; hold under nose).
- Open window for free circulation of air.

(2) Cyanmethemoglobin is the most stable of the various hemoglobin pigments showing no evidence of deterioration after 6 years of storage in a refrigerator. The availability of prepared standards is a distinct advantage of this technique. All hemoglobin derivatives are converted to cyanmethemoglobin with the exception of sulfhemoglobin.

(3) This method is highly accurate and is the most direct analysis available for total hemin or hemoglobin iron. Its disadvantage is the use of cyanide compounds which, if handled carefully, should present little hazard.

(4) For accuracy in hemoglobin determinations, it is absolutely necessary that the spectrophotometer and Sahli pipets be accurately calibrated.

(5) Venous samples give more constant values than capillary samples.

(6) If the procedure is performed properly, the degree of accuracy is ±2 to 3%.

h. Normal Values:

(1) Infants at birth: 18–27 g hemoglobin per dl.

(2) Childhood: 10–5 g hemoglobin per dl.

(3) Adult Males: 14–17 g hemoglobin per dl.

(4) Adult Females: 12–16 g hemoglobin per dl.

5-19. Detection of Hemoglobin S and Non S Sickling Hemoglobins:

a. Principle. Erythrocytes are introduced into a phosphate buffer solution containing a reducing agent and lytic agent. The red cells are lysed and the hemoglobin is reduced. Reduced sickling types of hemoglobin are insoluble in phosphate buffer and turbidity results. On addition of urea hemoglobin S dissolves.

b. Reagents:

(1) Stock Phosphate Buffer Solution. Dissolve 160.48 grams anhydrous potassium dihydrogen phosphate (KH_2PO_4), AR, and 28.88 grams anhydrous potassium monohydrogen phosphate (K_2HPO_4), AR in a 1-liter volumetric flash containing 500 ml of distilled water. Dilute to 1-liter with distilled water.

(2) Dithionite Reagent. Add 20.0 grams dithionite ($Na_2S_2O_4 \cdot 2H_2O$) and 0.25 grams saponin to a 100-ml volumetric flask. Add 80 ml of stock phosphate buffer solution. Mix well. Dilute to the mark with distilled water. This reagent remains stable under refrigeration at 4° C for approximately 1 month. WARNING. Sodium dithionite reagent may ignite if allowed to become damp. Use only clean dry utensils in handling.

(3) Urea (USP).

c. Procedure:

(1) Pipet 2 ml of dithionite reagent in 12 x 75 mm test tube.

(2) Add 0.02 ml of well-mixed anticoagulated blood (collected in EDTA).

(3) Mix the contents and allow to stand at room temperature for 5 minutes.

(4) After 5 minutes examine the tube for turbidity against a lined reader (see fig-

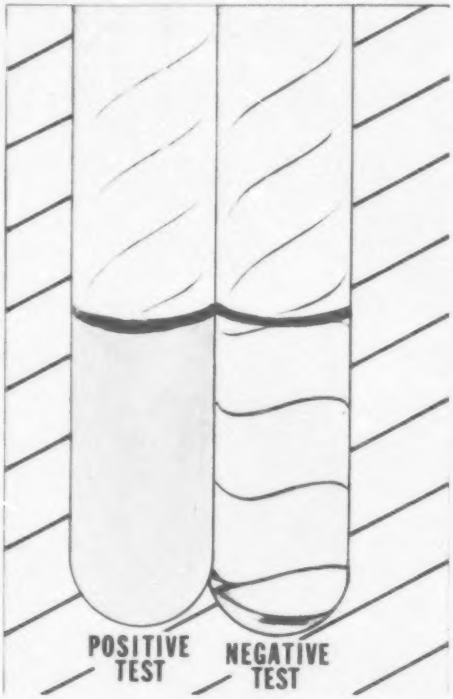


Figure 5-7. Dithionite Tube Test Interpretation.

ure 5-7). Hemoglobin S, if present, produces turbidity in the tube.

d. Sources of Error:

(1) The use of 10 x 75 mm test tubes could cause a false negative result.

(2) The dithionite reagent has a limited stability. The freshness of this reagent must be checked with positive and negative controls. The test should show the blue-pink color of reduced hemoglobin and adequate lysis of erythrocytes.

(3) Unstoppered tubes containing dithionite reagent decompose when left out at room temperature.

(4) False negative results could occur if the blood sample for testing is drawn within four months of transfusion.

e. Discussion:

(1) Hemoglobin S is an inherited type of hemoglobin found in Negroes and in people from Mediterranean areas.

(2) The degree of erythrocyte sickling is dependent on the concentration of hemoglobin. SS, SC, and SD cells sickle more rapidly than AS cells. Newborns with sickle cell anemia have erythrocytes more resistant to sickling due to the presence of hemoglobin F.

(3) The dithionite test also detects other sickling types of hemoglobin. Urea causes hemoglobin S (and structural variants of hemoglobin S) to dissolve. Other hemoglobins remain turbid in the presence of urea.

(4) This test is a rapid screening test for hemoglobin S. All positive tests should be electrophoresed for confirmation.

f. Interpretation. Hemoglobin S causes turbidity in the tube. Hemoglobin A is soluble in the phosphate buffer.

5-20. Demonstration of the Sickle Cell Phenomenon:

a. Principle. Erythrocytes of persons with sickle cell anemia or trait will assume a sickle shape when the oxygen tension is lowered. This may be demonstrated by mixing a drop of blood with a reducing agent such as sodium metabisulfite.

b. Reagent. Sodium Metabisulfite, 2%. Add 2 g of sodium metabisulfite to a 100-ml volumetric flask. Dilute to the mark with distilled water. This solution, if stored at 3° or 4° C remains effective for about 1 week.

c. Procedure:

(1) Place one drop of capillary (or venous without anticoagulants) blood on a clean glass slide.

(2) Add one or two drops of 2 percent aqueous sodium metabisulfite and mix.

(3) Place a coverglass on the preparation and express the excess blood by gently pressing the coverglass. The gentle pressure will produce a film thin enough to permit

examination of individual red cells. It is not necessary to seal the preparation.

(4) Observe immediately and at intervals of 15 to 30 minutes after preparation for signs of sickle cell information.

d. Discussion:

(1) The sickling phenomenon is a consequence of an inherited abnormal type of hemoglobin (hemoglobin S). The severity of the anemia as compared to the trait will vary with the proportion of defective to normal hemoglobin. This abnormal hemoglobin can be identified electrophoretically.

(2) When a fresh solution of sodium metabisulfite is used, positive cases should show 10 to 75 percent sickling within 15 minutes. There is a positive correlation between the degree of sickling and the severity of the disease.

(3) Normal blood treated in this way shows no sickling.

5–21. Hemoglobin Electrophoresis (Cellulose Acetate):

a. Principle. Hemoglobin fractions are separated by the rate of their protein migration in an electrical medium. The fractions are stained with Ponceau S and quantitated on a densitometer. The order of mobility from the cathode toward the anode is $A_3 > A_1 > F > S\text{–}D > C\text{–}A_2$.

b. Reagents:

(1) TEB Buffer (pH 8.8). Dissolve 16.5 g tris (hydroxymethyl) aminomethan (Tris), 1.56 g disodium EDTA and 0.92 g boric acid in 900 ml distilled water; dilute to 1-liter and mix. Store refrigerated in a polyethylene bottle. Discard when microbial growth appears or the pH changes.

(2) Sodium Chloride Solution, 0.9%. Add 9 g of sodium chloride (NaCl) to a 1-liter flask. Dilute to 1-liter with distilled water.

(3) Fixative-Dye Solution. Dissolve 0.2 g of Ponceau S, 3.0 g of trichloroacetic acid, and 3.0 g of sulfosalicylic acid in 100 ml of distilled water.

(4) Acetic Acid, 5%. Dilute 50 ml of glacial acetic acid to 1-liter with distilled water. Mix well and store in a glass-stoppered bottle.

(5) Clearing Solution (25% Cyclohexanone). Dilute 12.5 ml cyclohexanone to 50 ml with methanol; mix. Prepare fresh daily.

(6) Chloroform, AR.

c. Preparation of Hemolysate:

(1) Place 5 ml of blood into a tube containing an anticoagulant.

(2) Centrifuge and remove the plasma.

(3) Wash four times with 0.9% sodium chloride.

(4) Centrifuge, remove final wash, freeze 1 hour (keep frozen if specimen is to be analyzed at a later time), thaw, and add an equal amount of TEB buffer. Mix well.

(5) Let stand for 15 minutes at room temperature. Centrifuge at medium speed for 10 minutes. Transfer supernate to another tube; discard precipitate.

(6) Add an equal volume of chloroform, shake vigorously for 1 minute, and centrifuge.

(7) Decant the supernate. Determine the hemoglobin concentration of the hemolysate.

(8) Adjust to a value of 7 g hemoglobin per dl using an appropriate quantity of distilled water. The amount of distilled water to add to 1.0 ml of hemolysate is determined by the formula:

$$\text{ml Distilled water required} = \frac{\text{Conc. of original hemolysate}}{7} - 1$$

d. Procedure:

(1) Fill the cell with TEB buffer.

(2) Soak the cellulose acetate membrane in a tray of TEB buffer for 10 minutes. Gently blot the membrane to wipe off excess buffer.

(3) Place the membrane on the cell according to manufacturer's instructions.

(4) Apply the unknown and control hemolysates to the cathode side of the membrane.

(5) Electrophorese for 50 minutes at 375 volts.

(6) Immediately remove the membrane from the cell and transfer to a staining tray containing 50 ml of fixative-dye solution. Stain for 10 minutes.

(7) Rinse the membrane in three successive washes of 5% acetic acid. Drain excess liquid against the inside wall of the tray.

(8) Place a glass plate in a tray containing clearing solution. Transfer the membrane to the clearing solution for 1 minute. Agitate gently during clearing.

(9) Remove the glass plate from the clearing solution with the membrane positioned on the glass plate. Drain any clearing solution from the membrane and remove any air bubbles by squeezing. Dry the membrane at approximately 100° C for 10-15 minutes.

(10) Remove the plate from the oven and allow to cool. Place the plate in a humidity chamber for 30 minutes. Remove the membrane from the plate with a razor blade.

(11) Scan and integrate the area on a densitometer. (Follow the manufacturer's instructions for the operation of a densitometer.) Examples of scans are shown in figure 5-8.

(12) Report the hemoglobin genotype (AA, AS, AC, SS, CS, or CC) and the percent A_2 to the nearest tenth percent (0.1). An alkali denaturation test is also performed on all specimens and fetal hemoglobin reported. On all specimens migrating on S, a dithionite test is performed to rule out the possibility of its being a D. The dithionite test is not reported unless the results are inconsistent with S.

e. Calculations:

(1) Estimate the number of square millimeters for each fraction. Total the individual area.

(2) Calculate relative quantities of each fraction as follows:

$$\frac{\text{Fraction area}}{\text{Total area}} \times 100$$

f. Sources of Error:

(1) A heavy application of hemolysate

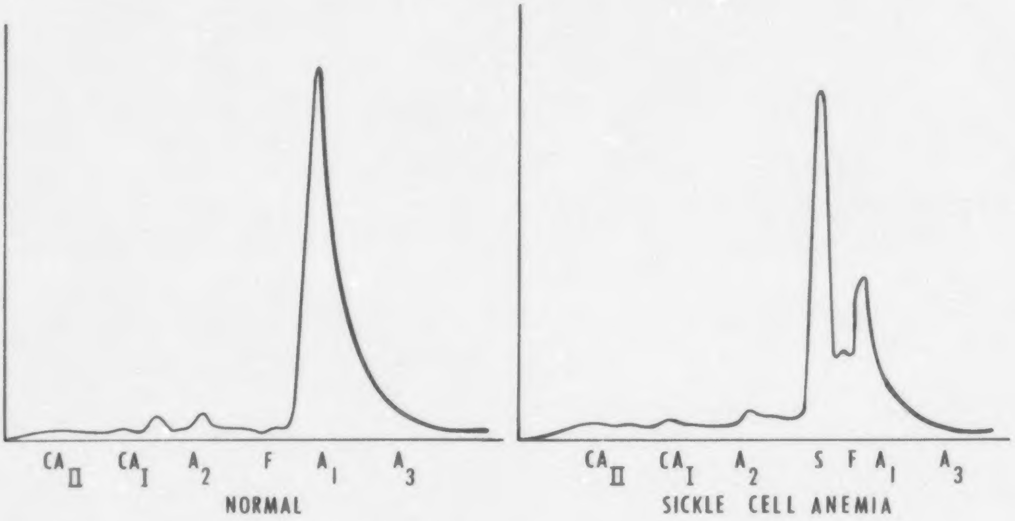


Figure 5-8. Hemoglobin Electrophoretic Scans.

causes a thick electrophoretic pattern after staining. Such a pattern must be repeated.

(2) Handling the cellulose acetate membrane with the hands interferes with the electrophoretic pattern. Always handle the membrane with tweezers.

(3) Some acetate membranes have defects. If any membranes are defective, discard the entire lot.

g. Discussion:

(1) A_2 hemoglobin migrates identically to hemoglobin C. They are distinguished by the quantity present. If this band is 40% or more of the total hemoglobin, it is C. A_2 hemoglobin should always be less than 20%.

(2) Two slow-moving, nonhemoglobin components are seen using this technique. These fractions are carbonic anhydrases I and II (CA_I and CA_{II}).

(3) Hemoglobin A_2 is elevated in thalassemia minor.

(4) Genotype SS is found in patients with sickle cell anemia.

(5) Genotype AS is found in patients with sickle cell trait.

(6) This method separates hemoglobin A_2 in the presence of hemoglobin S in patients manifesting sickle-thalassemi a disease.

(7) Hemoglobin F is quantitated by the alkali denaturation test because it migrates close to the hemoglobin A_1 fraction on the electrophoretic pattern.

(8) Include known A, S, and C controls in each analysis.

h. Normal Values:

(1) A_2 Hemoglobin: 1.5—3.4%.

(2) Genotype: AA.

(3) F Hemoglobin: 0—2% (except in infants).

5-22. Fetal Hemoglobin (Alkali Denaturation Test):

a. Principle. Fetal hemoglobin (HbF) is more resistant to denaturation in alkaline solution than adult hemoglobin (HbA). Alkali converts HbA to alkaline hematin. Alkaline hematin is insoluble and precipitates. HbF is quantitated by measuring the hemoglobin concentration before and after denaturation.

b. Reagents:

(1) Drabkin's Reagent. See paragraph 5-18b(1).

(2) Sodium Hydroxide (NaOH) 0.10 N. Dilute 5.55 ml saturated NaOH to 1-liter with distilled water. Mix well and store in a polyethylene bottle at room temperature.

(3) Ammonium Sulfate, Saturated. Add 400 g ammonium sulfate, AR, to 500 ml of distilled water. Stir mechanically for 10 minutes. Heat until all the salt has dissolved and filter rapidly through a Whatman #1 filter paper. Store in a polyethylene bottle.

c. Preparation of Hemolysate. See paragraph 5-21c.

d. Procedure:

(1) Dilute 0.2 ml of hemolysate with 1.8 ml of Drabkin's reagent and mix gently.

(2) Place 2.5 ml of 0.1 N NaOH in a 13 × 100 mm test tube. At zero time, add 0.5 ml of diluted hemolysate and mix rapidly.

(3) Exactly 2 minutes after addition of the hemolysate, add 2.0 ml of saturated ammonium sulfate. Mix by inverting six times. DO NOT SHAKE!

(4) Filter through a 7 or 9 cm Whatman #42 filter paper. If the filtrate is not crystal clear, filter again. This is the fetal hemoglobin solution.

(5) Prepare a total hemoglobin solution by adding 0.05 ml of diluted hemolysate to 5.0 ml distilled water. Mix well by inversion. DO NOT SHAKE!

(6) Prepare a fetal hemoglobin blank by adding 0.5 ml of Drabkin's reagent to 4.5 ml of distilled water. Mix as above.

(7) Prepare a total hemoglobin con-

trol by adding 0.05 ml of Drabkin's reagent to 5.0 ml of distilled water. Mix as above.

(8) Measure the absorbances of the fetal and total hemoglobin solutions against the appropriate blanks.

e. Calculations:

$$\frac{\text{Absorbance fetal hemoglobin solution}}{\text{Absorbance total hemoglobin solution}} \times 10 = \%\ \text{fetal hemoglobin}$$

Report % to nearest tenth (0.1).

f. Sources of Error:

(1) If the hemoglobin concentration of the hemolysate is less than 5 g per dl, the sensitivity of the procedure is decreased.

(2) Poor pipetting technique causes a significant error due to the small volumes involved.

(3) If the absorbance of the fetal hemoglobin solution is greater than 0.700, dilute 1.0 ml of the fetal hemoglobin solution with 9.0 ml distilled water. Read the absorbance against the total hemoglobin blank. Multiply the calculated result by 10.

g. Discussion:

(1) The alkali denaturation test can be performed on bloody rectal discharges from infants. This test is used to determine whether the discharge is due to ingested maternal blood or a gastrointestinal lesion. The procedure is as follows:

(a) Add a small amount of bloody discharge to 10 ml of distilled water.

(b) Add 0.2 ml 10% sodium hydroxide solution.

(c) If the solution turns pink, it is fetal blood. A muddy brown solution indicates blood of maternal origin.

(2) Fetal hemoglobin constitutes approximately 80% of a newborn infant's hemoglobin. This decreases to approximately 5% at six months.

(3) Fetal hemoglobin is increased in adults with thalassemia major and minor, hereditary persistence of HbF, erythroleukemia, aplastic anemia, and spherocytosis.

h. Normal Values:

(1) Infants (less than 6 months): up to 80%.

(2) Adults: 0—2%.

SECTION E—EXAMINATION OF BLOOD SMEARS

5-23. Introduction:

a. The critical examination of a blood smear includes the following: quantitative and qualitative study of platelets, differential count quantitating the three types of leukocytes (granulocytes, lymphocytes, monocytes), and morphological characteristics of erythrocytes and leukocytes. Staining the blood smears is a critical part of the examination. The procedure for staining is described in chapter 3. To accurately perform the differential count it is necessary for a technician to recognize all the characteristics of normal blood cells. This includes normal biological variation. For instance, not every lymphocyte is exactly the same size, nor do all lymphocytes have exactly the same number azurophilic granules.

b. Certain morphological and histochemical characteristics are utilized to differentiate blood cells. A review of the significant features promotes a better understanding of blood differentials. Cellular characteristics such as relative size, shape, cytoplasmic granulation, nuclear-cytoplasmic ratio, nuclear configuration, chromatin or nucleoli are very important. These features are discussed in chapter 4.

c. Experience is the foremost teacher in hematology. It is readily acquired in a busy hematology section where the opportunity for differential analysis occurs frequently. Experience can be diversified and interesting if proficiency slides and material from cases of confirmed diagnoses are maintained as study sets. This study material should be available to all technicians in the laboratory.

d. All routine blood smears should be

kept until the physicians have reviewed the differential reports. A 1-week period is usually adequate. Occasionally, a review of a specific problem slide results in findings which were not originally apparent and reinforces confidence in the laboratory by the medical staff. This practice also adds to the experience and proficiency of the technician.

5-24. Examination of Peripheral Blood Smears:

a. Principle. The stained blood smear permits the study of the appearance and the identification of the different kinds of leukocytes, and the appearance of erythrocytes and thrombocytes (blood platelets).

b. Differential Leukocyte Count:

(1) Inspect the smear under low power magnification. Locate the thin end of the smear where there is no overlapping of erythrocytes.

(2) Switch to oil immersion. Identify and count 100 consecutive leukocytes and record each cell type separately on the differential counter. Begin at the thin end of the smear and count the white cells observed as the slide is moved in a vertical direction. When near the edges of the smear, move the slide horizontally for a distance of about two fields, then proceed vertically back across the smear. See figure 5-9. Continue this "snake-like" movement until 100 leukocytes have been counted and classified.

(3) If the WBC count is between 20,000 and 50,000 per cu mm of blood, count and classify 300 leukocytes. When the count is greater than 50,000 per cu mm of blood, count and classify 500 leukocytes.

(4) The number of each type of leukocyte is expressed as a percent of the total number of white cells counted. Absolute values may be calculated by multiplying the percent value by the total leukocytic count.

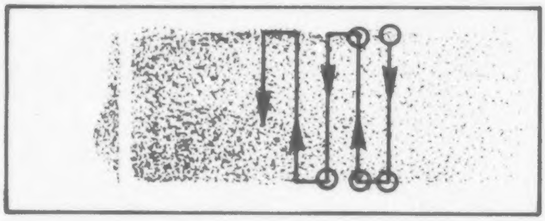


Figure 5-9. Examination of Peripheral Blood Smears.

c. Erythrocyte Morphology:

(1) Study the erythrocytes and report any evidence of rouleaux formation or signs of immaturity.

(2) Report the erythrocyte morphology with reference to size, shape, staining characteristics, and inclusions. Report the degree of the specific morphological characteristic (for example, moderate hypochromia).

(3) If nucleated erythrocytes are found (usually these are metarubricytes), report the number per 100 leukocytes counted.

d. Qualitative Platelet Evaluation:

(1) Observe the thrombocytes in several oil immersion fields to obtain a rough estimation of their numbers (normal, increased, or decreased). Normal is an average of 4-6 per oil immersion field.

(2) Note any abnormality in morphology (giant platelets, etc). If the thrombocytes appear to be significantly decreased, a thrombocyte count and/or a clot retraction test may be indicated.

e. Discussion:

(1) All abnormal white cells (for example, immature, hypersegmented, toxic,

atypical lymphocytes, etc.) should be classified or described and reported in percent, separately. Cells that are ruptured, fragmented, or degenerated are not included in the differential count, but should be noted separately and reported as the number seen per 100 leukocytes.

(2) In view of the gradual transition from the metamyelocyte to the band neutrophil and then to the segmented neutrophil, exact classification is sometimes difficult. In such cases, classify the cell according to the more mature form.

(3) Size considerations in differentiating blood cells require a defined linear standard. The micron (.001 mm) is usually used in reference to microscopic dimensions. Ocular micrometers are available through Federal medical supply channels and are easily calibrated, using a hemacytometer which has standardized dimensions. In routine screening of blood smears, an experienced technician relates the size of a normocytic erythrocyte (seven to eight microns) to the size of the white cell to be differentiated, since erythrocytes are usually present throughout the microscopic field. Finally, it should be understood that personal visual discrimination is an inaccurate gauge of linear measure. Some reference measure should be employed.

(4) The shape of blood cells often depends upon the smear and staining technique. Variations that have no clinical significance occur from physical and chemical distortion that result from technical error. These variations are avoided with careful technique. Each routine smear should be scanned initially to evaluate the smear and stain quality before differential analysis.

(5) Cytoplasmic granulation—neutrophilic, basophilic or eosinophilic—is an important morphological observation. Differences in granule color in Wright-stained preparations are caused by the variable dye affinity of specific granules. The intensity of colors and the relative blueness or redness of the erythrocytes is used to evaluate the quality of the stain. The familiar basophilic (blue), eosinophilic (red), and neutrophilic (pink) granules are quite obvious in routine blood smears. The presence, absence, type, and quantity of granules are characteristic attributes used to differentiate leukocytes.

(6) The size ratio of nucleus to cytoplasm (N:C) is a differentiating characteristic. For instance, a cell with a nuclear mass equal to the cytoplasmic mass would have an N:C ratio of 1:1. The total cell mass is usually greater in the more immature cells and decreases as the cell matures. The nuclear mass usually decreases also as the cell matures. Of course, lymphocytes are the exception to this generality.

(7) The nuclear configurations of leukocytes help distinguish these cells. Round, oval indented, band, or segmented are terms used to describe variations in shape. These normal configurations can be distorted by physical and chemical factors. Some of the leukocytes are so fragile that in thick blood smears their normal configuration may be distorted by the pressure of erythrocytes forced against them. These artifacts should be recognized as such in an intelligent evaluation of blood differentials.

(8) In addition to nuclear shape and size, the internal nuclear morphology shows differential inclusions. The chromatin appears finely reticulated in some cells, or as a coarse network, or even clumped, in others. The parachromatin, a lighter staining material beside the chromatin, is scant or abundant. The appearance of the chromatin and the quality of parachromatin are utilized to differentiate blood cells. The presence, absence, and number of nucleoli in the nucleus are the most distinctive characteristics of immature nuclei in blood cells.

(9) All abnormal blood smears should be examined by another trained person for confirmation of the results.

f. Normal Differential Values:

(1) Neutrophilic metamyelocytes: 0-1%.

(2) Neutrophilic bands: 3–5%.

(3) Segmented neutrophils: 55–65%.

(4) Eosinophils: 2–4%.

(5) Basophils: 0–1%.

(6) Lymphocytes: 20–35%.

(7) Monocytes: 2–6%.

5-25. Examination of Blood Marrow Smears:

a. Principle. Nucleated blood cells are counted and classified from a bone marrow smear stained with Modified Wright's stain.

b. Procedure:

(1) Using oil immersion magnification, count and classify 300–500 nucleated cells.

(2) Classify all blood cells according to cell type and various stages of maturation.

(3) Calculate myeloid-erythroid ratio by dividing the number of nucleated erythrocytes into the number of granulocytic (myeloid) cells.

(4) A peripheral blood evaluation usually accompanies the bone marrow reports. This evaluation usually includes an erythrocyte count, leukocyte count, differential count, hemoglobin, hematocrit and a reticulocyte count.

c. Discussion:

(1) The differential cell count on a bone marrow smear is carried out by a hematologist, pathologist, or trained technician.

(2) Since interpretation of findings in bone marrow examinations is very difficult, it is of utmost importance that the smears and stains are carefully prepared using scrupulously clean equipment.

(3) The laboratory technician is usually responsible for preparing bone marrow smears, staining the smears, checking the quality of the stained smear, and coverslipping the slides.

d. Normal Values:

(1) Leukocytes:

(a) Myeloblasts: 0–5%.

(b) Promyelocytes: 2–8%.

(c) Neutrophilic myelocytes: 4–16%.

(d) Neutrophilic metamyelocytes: 5–20%.

(e) Neutrophilic bands: 10–35%.

(f) Neutrophilic segmented cells: 7–30%.

(g) Eosinophilic cells: 1–4%.

(h) Basophilic cells: 0–1%.

(i) Lymphocytes: 5–15%.

(j) Monocytes: 0–5%.

(k) Plasmocytes: 0–1%.

(2) Erythrocytes:

(a) Rubriblasts: 0–1%.

(b) Prorubricytes: 1–4%

(c) Rubricytes: 3–10%.

(d) Metarubricytes: 5–25%

(3) Megakaryocytes: 0–3%.

(4) Myeloid-Erythroid Ratio (M:E): 3–4:1.

SECTION F—ERYTHROCYTE INDICES AND FRAGILITY TESTS

5-26. Erythrocyte Indices:

a. Principle. By using accurately determined red blood cell counts, hemoglobin and hematocrit values, the size and hemoglobin content of the average red cell in a given blood sample is calculated. The values obtained are the erythrocyte indices and aid in the classification and study of anemias.

b. Calculation of Erythrocyte Indices.

(1) Mean Corpuscular Volume (MCV) —The average volume of the individual red blood cell.

$$\frac{\text{Hematocrit (percent)} \times 10}{\text{Red cell count (in millions)}} = \text{cubic microns}$$

Example: Hematocrit 45 percent
Red count 5,000,000 per cu mm

$$\frac{45 \times 10}{5.0} = 90 \text{ cubic microns (normal)}$$

(2) Mean Corpuscular Hemoglobin (MCH)—The average weight of hemoglobin of the individual red cell.

$$\frac{\text{Hemoglobin (gm per dl)} \times 10}{\text{Red cell count (in millions)}} = \text{micromicrograms}$$

Example: Hemoglobin 15 gm per dl
Red count 4,000,000 per cu mm

$$\frac{5 \times 10}{5.0} = 30 \text{ micromicrograms (normal)}$$

(3) Mean Corpuscular Hemoglobin Concentration (MCHC)—The percent of hemoglobin in the average red cell.

$$\frac{\text{Hemoglobin (gm per dl)} \times 100}{\text{Hematocrit (percent)}} = \text{percent}$$

Example: Hemoglobin 15 g per dl
Hematocrit 45 percent

$$\frac{15 \times 100}{45} = 33 \text{ percent (normal)}$$

c. Discussion:

(1) Accurate individual determinations of hemoglobin, hematocrit, and erythrocyte count insure reliable indices. The following procedures are recommended:

(a) Erythrocyte count—two separate pipets and 2-4 counting chambers or electronic cell counting.

(b) Hemoglobin—precise reagent standards and accurate instrument calibration.

(c) Hematocrit—Wintrobe, centrifuge at 3,000 rpm for 55 minutes.

(2) It is useful to compare the calculated indices with a stained peripheral blood smear.

(3) Wintrobe classified anemias into the following groups on the basis of the indices:

Anemia	*MCV*	*MCH*	*MCHC*
Macrocytic	95–160	32–50	32–36
Microcytic	72–79	21–24	24–36
Microcytic Hypochromic	50–79	19–29	24–30
Normocytic	80–94	26–32	32–36

(4) The MCHC cannot exceed the normal value, since the erythrocyte cannot be supersaturated with hemoglobin. The MCHC is the most valid of the indices since it does not require the erythrocyte count in its derivation. It is a good index of iron deficiency.

(5) The MCV and MCH are increased at birth and fall to low values during childhood. The MCHC is fairly constant for all ages.

d. Normal Values:

(1) Mean Corpuscular Volume: 82–92 cubic microns.

(2) Mean Corpuscular Hemoglobin: 27–31 micromicrograms.

(3) Mean Corpuscular Hemoglobin Concentration: 32–36%.

5-27. Erythrocyte Osmotic Fragility Test (Dacie):

a. Principle. A specific amount of blood is introduced into a series of tubes containing different concentrations of buffered salt solutions. The ability of the erythrocytes to resist hemolysis in such solutions is determined spectrophotometrically on the basis of free hemoglobin present.

b. Reagents:

(1) Buffered Saline (Equivalent to 10% NaCl). Add 180 g of dry sodium chloride (NaCl), 27.31 g mono-hydrogen sodium phosphate (Na_2HPO_4), and 4.86 g dihydrogen sodium phosphate (NaH_2PO_4) to a 2-liter volumetric flask. Dilute to the mark with distilled water.

(2) Working Solution (1% NaCl). Add 10 ml of the buffered saline to a 100-ml volumetric flask. Dilute to the mark with distilled water.

c. Procedure:

(1) Set up two series of 12 tubes in a rack; label one series PATIENT and the other CONTROL.

(2) Set up the following dilutions:

Tube Number	*Working Solution*	*Distilled Water*	*NaCl Concentration*
1	4.25 ml	0.75 ml	0.85%
2	3.75 ml	1.25 ml	0.75%
3	3.25 ml	1.75 ml	0.65%
4	3.00 ml	2.00 ml	0.60%
5	2.75 ml	2.25 ml	0.55%
6	2.50 ml	2.50 ml	0.50%
7	2.25 ml	2.75 ml	0.45%
8	2.00 ml	3.00 ml	0.40%
.9	1.75 ml	3.25 ml	0.35%
10	1.50 ml	3.50 ml	0.30%
11	1.00 ml	4.00 ml	0.20%
12	0.50 ml	4.50 ml	0.10%

(3) Draw 5 ml of venous blood from the patient and deliver into a tube containing heparin.

(4) Pipet 0.05 ml blood into each tube of the PATIENT series. Mix immediately.

(5) Similarly collect 5 ml of venous blood from a normal individual and deliver into a tube containing heparin. Pipet 0.05 ml of blood into each tube of the CONTROL series. Mix immediately.

(6) Allow the tubes to stand at room temperature for 30 minutes.

(7) Mix again and centrifuge at 2,000 rpm for 5 minutes.

(8) Transfer the supernates to cuvets and read at 545 nm using tube number 1 as a blank and number 12 as 100% hemolysis.

(9) Read and record the absorbances for each tube of the CONTROL series and each tube of the PATIENT series.

(10) Calculate the percent hemolysis at each salt concentration for both series of tubes.

(11) Plot the curves of the patient's and control's osmotic fragility using the percent hemolysis as the vertical ordinate and the salt concentration as the horizontal abscissa (see figure 5–9 for an example curve).

d. Calculation:

$$\%\ \text{Hemolysis} = \frac{\text{Absorbance of a particular tube}}{\text{Absorbance of tube 12}} \times 100$$

e. Sources of Error:

(1) The concentration of the NaCl in the solutions is critical. The salt must be chemically pure and dried before weighing. It is advisable to dry the salt in a 100° C oven and store it in a desiccator. Store the NaCl solutions in a glass-stoppered tightly sealed bottle.

(2) Inaccurate preparation of the dilutions causes inaccurate results.

(3) Maintain the pH of the solution at an interval of 7.35–7.50. A different pH range causes invalid results.

(4) Rough handling of the blood specimen causes hemolysis which leads to invalid results.

f. Discussion:

(1) In hypotonic salt solutions, erythrocytes take up water, swell to a spheroid shape and burst. In congenital spherocytic anemia the red cells with defective structure more readily rupture at salt concentrations closer to isotonicity (0.85 percent). These cells thus show an increased osmotic fragility. In contrast, the flat or thin but otherwise normal red cells of hypochromic anemia show a decreased osmotic fragility and do not hemolyze until lower salt concentrations are reached.

(2) When hemolysis begins beyond the range of the prepared solutions or when intermediate dilutions are desired, the additional dilutions are readily prepared using the 1 percent sodium chloride stock solution.

(3) In cases where the results of the fragility test are borderline, the following procedure is recommended to enhance any latent abnormality in fragility. Incubate samples of defibrinated blood (control and patient's) at 37° C for 24 hours under sterile conditions and controlled pH (7.35–7.50). The test is then performed as described above.

(4) Decreases in pH increase osmotic fragility. The reagents are buffered to maintain a constant pH of 7.35–7.50.

(5) This test may also be run visually, with some sacrifice of accuracy, by allowing the blood-saline dilutions to stand at 20° C for 45 minutes. The tubes are then lightly centrifuged (1,000 rpm for 3 minutes) and observed for signs of initial and complete hemolysis. A slight pink coloration of the supernatant fluid indicates initial hemolysis and a clear red solution, free of sediment, indicates complete hemolysis. The salt concentrations in these two tubes are noted and recorded. The control should always be reported along with results of patient's tubes.

g. Normal Values:

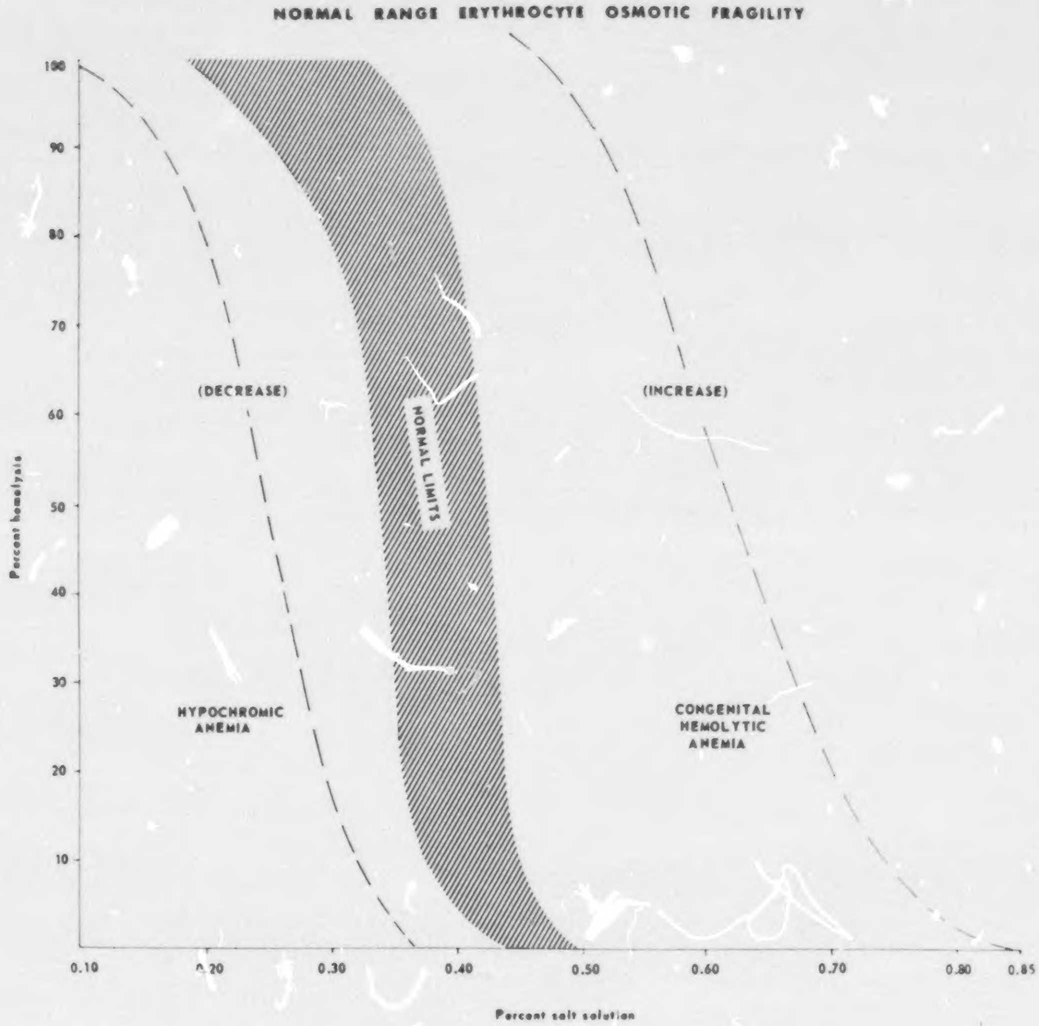


Figure 5-10. Erythrocyte Osmotic Fragility Curve.

(1) 0.30% Saline: 97–100% hemolysis.
(2) 0.35% Saline: 90–99% hemolysis.
(3) 0.40% Saline: 50–95% hemolysis.
(4) 0.45% Saline: 4–45% hemolysis.
(5) 0.50% Saline. 0–6% hemolysis.
(6) 0.55% Saline: 0% hemolysis.

5-28. Ham Test for Erythrocyte Fragility:

a. Principle. This test is positive in paroxysmal nocturnal hemoglobinuria (PNH). Erythrocytes in this form of anemia lyse easily in slight variations in the pH (acid). In this test, the erythrocytes are subjected to pH values ranging from 6.5 to 7.0 at 37° C.

b. Reagents:

(1) Hydrochloric Acid, 0.33 N. Add 29 ml concentrated HCl to a 100-ml volumetric flask. Dilute to the mark with distilled water. Mix.

(2) Sodium Chloride Solution (Saline),

0.9%. Add 9 grams sodium chloride to a 1-liter volumetric flask. Dilute to the mark with distilled water.

c. Procedure:

(1) Carefully withdraw and defibrinate 10 ml of venous blood from the patient.

(2) Carefully withdraw and defibrinate 10 ml of venous blood from a normal individual.

(3) Centrifuge both blood specimens at 1,000 rpm for 10 minutes.

(4) Separate serum and cells.

(5) Wash the cells three times with normal saline, then prepare a 5 percent suspension of cells in normal saline.

(6) Measure 1.0 ml of the 5 percent test cells into each of 4 tubes. Repeat this for the control normal specimen.

(7) Acidify 1.9 ml of serum by adding 0.1 ml of 0.33 N hydrochloric acid. Do this for both the test and control serums.

(8) Into each of the tubes add test serum, control serum, acidified serum, and acidified test serum as follows:

Test	*Control*	*Add:*
1	1	1.0 ml test serum
2	2	1.0 ml control serum
3	3	1.0 ml acidified test serum
4	4	1.0 ml acidified control serum

(9) Mix all eight tubes and incubate for 1 hour at 37° C. Shake every 10 minutes.

(10) Centrifuge at the end of the 1-hour period and examine for hemolysis.

d. Discussion:

(1) With a positive test the tubes containing acidified sera and patient's cells should show considerable hemolysis.

(2) Normally no tubes should show hemolysis.

(3) Occasionally, tubes with unacidified sera and patient's cells may show moderate hemolysis.

(4) A false positive test is sometimes seen in congenital spherocytic anemia.

(5) If congenital spherocytic anemia is suspected, the test should be repeated, using acidified serum previously inactivated at 56° C for 30 minutes.

(6) Since erythrocytes of PNH require complement for hemolysis, the modified test (item 5 above) will be negative in PNH and will remain positive in spherocytosis.

e. Interpretation. Hemolysis in the acidified tube is indicative of paroxysmal nocturnal hemoglobinuria.

SECTION G—DEMONSTRATION OF L.E. CELLS

5-29. General Information:

a. Persons having lupus erythematosus, one of the "collagen" diseases, have an abnormal plasma protein which causes swelling and breakdown of certain blood cell nuclei in vitro. This degenerated nuclear material attracts phagocytic cells, particularly segmented neutrophils, which engulf this nuclear mass. The resulting phagocyte and inclusion material is termed an "L.E." cell.

b. The two most popular methods of demonstrating the L.E. cell and antinuclear antibodies are the rotary bead method and fluorescent antibody method. The rotary bead method is positive in 75–80% of the patients with lupus erythematosus. The fluorescent antibody method is positive in 95–100% of the patients with lupus erythematosus. The rotary bead method is presented in the next paragraph. The fluorescent antibody method requires equipment that limits its use to larger laboratories.

5-30. Rotary Bead Method:

a. Principle. Leukocytes are broken down in vitro allowing the abnormal plasma protein to react on the altered nuclear material. Incubation enhances the nuclear deterioration and phagocytosis. Slides are prepared and examined for the peculiar "L.E." cell.

b. Procedure:

(1) Place 10 glass beads and 100 units of sodium heparin (U.S.P.) in a 15 by 125 mm test tube.

(2) Obtain 10-15 ml of venous blood from the patient and place in the prepared tube.

(3) Mix the blood and anticoagulant gently three-four times.

(4) Let the blood specimen incubate at room temperature for 1 hour.

(5) Place on any suitable hematological sample mixer and rotate at a slow speed (12-15 rpm) for 30 minutes.

(6) Place in centrifuge and spin down at 2,000 rpm for 15 minutes.

(7) Remove and discard supernatant plasma.

(8) With a disposable pipet remove the buffy coat and a small amount of RBCs and place in a Kahn tube.

(9) Mix well and fill three nonheparinized capillary tubes with the mixture.

(10) Spin capillary tubes down in a microhematocrit spinner for 4 minutes.

(11) Etch the capillary tubes with a diamond point pencil at the juncture of the RBC-buffy coat layer and break the tube.

(12) Tap out the buffy coat and a small amount of plasma on a slide and mix until the cells are homogeneous. Smear the cells out in the same manner as for a differential smear and let air-dry. Prepare two smears.

(13) Stain the smears with modified Wright's stain and examine the smears under oil immersion for the presence of the lupus erythematosus phenomenon.

(14) Examine known positive slides along with the patient slides.

c. Discussion:

(1) Lupus erythematosus is a chronic, sometimes fatal, disease of unknown etiology. The peculiar skin eruption across the nose and cheeks (butterfly rash) and arthritis can be accompanied by various visceral manifestations. Often the rash is not present, and diagnosis depends on demonstration of the L.E. cell. Frequently the earliest symptoms appear after intense exposure to sunlight. Leukopenia, thrombocytopenia, and an elevated sedimentation rate are some of the clinical signs of the disease.

(2) Free masses of lysed nuclear material, with or without polymorphonuclear leukocytes clustered about them (rosette formation), are suggestive of the L.E. phenomenon. Observing "rosettes" should encourage the technician to repeat examinations and further search for the true "L.E." cells. A positive report should not be made without the identification of this cell. The inclusion body with the leukocyte is homogeneous and has no chromatin pattern. This feature distinguishes the true "L.E." cell from the "tart" cell (nucleophagocytosis). This latter cell contains an engulfed, damaged nucleus, usually that of a lymphocyte which still contains a recognizable chromatin pattern and a distinct nuclear membrane.

d. Interpretation:

(1) These cells are seen as large polymorphonuclear (segmented) leukocytes which contain large ingested nuclear fragments in their cytoplasm.

(2) The inclusion body is a purplish-staining, smoky, homogeneous mass of material which is so large that it usually pushes the nucleus to one side of the cell.

SECTION H—SPECIAL STAINS

5-31. Peroxidase Stain (Kaplow):

a. Principle. The members of the granulocytic series contain an enzyme, peroxidase, which liberates the oxygen from hydrogen peroxide. This enzyme is more prominent in mature forms. A benzidine derivative is used as an indicator of peroxidase activity. The indicator is oxidized and precipitates in the form of brown to blue granules.

b. Reagents:

(1) Formalin-Ethanol Solution, 10%.

Mix 10 ml of formaldehyde with 90 ml of absolute ethanol.

(2) Stain. Mix the following reagents in the order listed:

30% Ethanol	100.0	ml
Benzidine dihydrochloride	0.3	g
0.132 M $ZnSO_4 \cdot 7H_2O$ (3.8% W/V)	1.0	ml
Sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$)	1.0	g
3% Hydrogen peroxide	0.7	ml
1.0 M sodium hydroxide	1.5	ml
Safranin O	0.2	g

Mix well after each addition. Final pH is 6.0 ±0.05. Store the stain in a screw-capped jar. The stain is stable for about six months.

c. Procedure:

(1) Prepare blood smears from fresh blood or bone marrow aspirates. DO NOT use anticoagulated blood.

(2) Fix the smears in 10% formalin-ethanol solution for 60 seconds.

(3) Wash off fixative gently for 15–20 seconds in running water. Shake off excess water.

(4) Place the slides in a Coplin jar containing the stain for 30 seconds at room temperature.

(5) Wash the slides for 5–10 seconds in running water and dry. Examine the smears under oil immersion.

d. Interpretation. Peroxidase positive cells are identified by yellow-green to blue and brown-green granules. Cells of the granulocyte series from the promyelocyte through the segmented neutrophil are peroxidase positive. The degree of peroxidase activity increases as the granulocytes mature. Monocytes may show a weak reaction. All other cells are negative.

e. Discussion:

(1) The oxidizing enzyme in the granules of the granulocytic leukocytes deteriorates rapidly in vitro. It is, therefore, necessary to use fresh blood in making the preparation.

(2) Smears should be prepared within 1 hour of obtaining the specimen and stained within 3 hours after they are prepared.

(3) The monocyte is thought to be slightly peroxidase positive through the phagocytization of peroxidase positive granules of ruptured cells.

(4) Myeloblasts can show weak peroxidase activity using this method.

(5) Addition of 4.9 mg of sodium cyanide to the stain inhibits peroxidase activity in all granulocytes except eosinophils.

(6) If greater nuclear detail is required, counterstain with 1% aqueous cresyl violet acetate for 1 minute or in freshly prepared Giemsa stain for 10 minutes.

(7) Giemsa stain is prepared as follows: Mix 3.8 g Giemsa stain powder and 200 ml glycerin. Incubate at 60° C for two hours. Add 312 ml absolute methanol; dilute the staining solution 1:10 with 1% sodium carbonate before use.

5–32. Leukocyte Alkaline Phosphatase (Kaplow):

a. Principle. Blood smears are fixed and stained for alkaline phosphatase activity.

b. Reagents:

(1) Fixative Solution. Mix 10 ml formaldehyde and 90 ml absolute methyl alcohol. Store in the freezing unit of a refrigerator.

(2) Propanediol Stock Solution, 0.2 M. Add 10 g 2-amino-2-methyl-1, 3-propanediol in a 500-ml volumetric flask. Dissolve in, and dilute to the mark with, distilled water. Store in the refrigerator.

(3) Propanediol Buffer, 0.05 M, (pH 9.75). Add 25 ml 0.2 M propanediol stock solution and 5 ml of 0.1 M hydrochloric acid to a 100-ml flask. Dilute to the mark with distilled water. Store in the refrigerator.

(4) Substrate Mixture. Dissolve 20 mg naphthol AS–BI phosphate (Sigma Chemical Company) in 1.0 ml of dimethylformamide. Add 250 ml of 0.05 M propanediol buffer to the solution. Pipet 10 ml aliquots of

this solution into test tubes, stopper and freeze.

(5) Stain Solution. Thaw substrates tubes to room temperature (as needed). Add 4 mg of fast red-violet salt LB (Sigma Chemical Company) to each 10 ml of substrate solution. Mix vigorously for 3 seconds, filter and USE IMMEDIATELY.

(6) Counterstain (Harris Hematoxylin):

(a) Dissolve 100 g of potassium or ammonium alum in 1000 ml of distilled water. Heat until the solid dissolves.

(b) Dissolve 5 g of hematoxylin crystals in 50 ml of 95% ethyl alcohol.

(c) Mix the alum and hematoxylin solutions. Heat to boiling and add 2.5 g mercuric oxide.

(d) Reheat the solution for about 1 minute until dark purple.

(e) Rapidly cool in cold water.

(f) This stain is stable at room temperature for 3 to 4 months.

c. Procedure:

(1) Prepare fresh blood smears and air-dry.

(2) Prepare negative controls from normal human blood.

(3) Positive smears are obtained by collecting blood smears from obstetric patients during the first 24 hours after delivery.

(4) Immerse the slides in the fixative 0° to −10° C for 30 seconds.

(5) Gently wash the smears in running water and air-dry.

(6) Place the slides in the stain solution for 10 minutes at room temperature.

(7) Repeat step (5).

(8) Counterstain with hematoxylin stain for 3 to 8 minutes.

(9) Repeat step (5).

(10) Mount with glycerin gelatin or Permount (Fisher Scientific Company) and examine under oil immersion.

d. Scoring:

(1) Count two slides (100 cells per slide) on each patient, rating the segmented neutrophils according to how much black staining of the granules is observed. If no staining is noted, the rating is 0; if slight black staining is noted, the rating is 1+, if a medium amount of black staining is noted, the rating is 2+, if a heavy amount of dark black staining is observed, the rating is 3+, and if there is heavy black staining covering all the cytoplasm, the rating is 4+.

(2) After 100 cells per slide are rated, figure the score-giving cells counted as 0—no score; cells rated as 1+ get a score of 1 each; cells rated as 2+ get a score of 2 each, etc.

(3) Report the total number of cells, giving their ratings and score. Report the total score for each individual slide. Average the two total scores and report the average. Also, always report the normal score range.

d. Discussion:

(1) Patients with infections, polycythemia and myeloproliferative disorders demonstrate increased alkaline phosphatase activity.

(2) In patients with acute or chronic granulocytic leukemia, alkaline phosphatase activity is decreased.

f. Normal Values. Scores of 13–130 have been obtained in healthy adults. However, the attending physician should interpret whether values are normal or abnormal.

5–33. Heinz Body Stain:

a. Principle. Blood is mixed with methyl violet solution and a smear is prepared. Heinz bodies stained with methyl violet are purple, round or oval granules, one-two microns in diameter within the erythrocytes.

b. Reagent. Methyl Violet Solution. Dissolve 0.5 g methyl violet in 100 ml of saline (0.9% NaCl). Filter.

c. Procedure:

(1) Mix equal volumes of blood and

methyl violet solution in leukocyte diluting pipet.

(2) Let stand at room temperature for 15 minutes.

(3) Prepare a smear and examine under oil immersion.

d. Discussion:

(1) Heinz bodies are invisibile in Wright-stained preparation.

(2) They can be observed in reticulocyte preparations and by the use of phase microscopy.

(3) Heinz bodies are thought to be denatured hemoglobin. They are usually demonstrated in hemolytic anemias caused by toxic agents.

5–34. Siderocyte Stain:

a. Principle. Siderocytes are erythrocytes containing iron granules. The granules are blue when stained with Prussian blue.

b. Reagents:

(1) Prussian Blue. Dissolve 4 g of potassium ferrocyanide in 20 ml distilled water. Add concentrated HCl until a white precipitate forms. Filter to remove the precipitate.

(2) Safranin Solution, 1%. Add 0.1 (100 mg) g safranin to 10 ml distilled water.

c. Procedure:

(1) Cover blood or bone smears with Prussian blue for 30 minutes (NOTE: Wright-stained preparation can be used).

(2) Wash the Prussian blue off the slide with distilled water.

(3) Counterstain with 1% safranin solution for 2–3 seconds.

d. Interpretation. Iron granules present in erythrocytes stain blue.

e. Discussion:

(1) Siderocytes occur in several anemias, lead poisoning, and after splenectomy.

(2) On Wright-stained preparation the granules are bluish-purple and are called Pappenheimer bodies.

SECTION I—MISCELLANEOUS TESTS

5–35. Screening Test for Glucose-6-Phosphate Dehydrogenase Deficiency:

a. Principle. Blood is oxygenated to convert hemoglobin to oxyhemoglobin. Sodium ascorbate in the presence of oxyhemoglobin yields hydrogen peroxide. In G-6-PD deficient individuals, hydrogen peroxide reacts with hemoglobin to form a brown to green heme chrome.

b. Reagents:

(1) Glucose Sodium Ascorbate. Mix 1.0 g glucose and 2.0 g sodium ascorbate in a mortar. Transfer 15 mg of the mixture to a series of 16 x 100 mm test tubes and stopper. These tubes are stable indefinitely when stored at −20° C.

(2) Phosphate Buffer, 1/15 M (pH 7.4). Dissolve 0.9 g potassium dihydrogen phosphate (KH_2PO_4) and 3.81 g sodium monohydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$) in distilled water to make 500 ml.

(3) Sodium Cyanide Solution. Dissolve 500 mg sodium cyanide in approximately 50 ml distilled water. Add 20 ml 1/15 M phosphate buffer. Add 1 M HCl until the pH is 7.4 (about 10 ml). Dilute to 100 ml with distilled water.

c. Procedure:

(1) Collect a blood sample in EDTA or heparin from the patient and a normal individual.

(2) Aerate the blood samples to a bright red color by gentle swirling under air.

(3) Add 2 ml aerated blood to each tube containing the glucose sodium ascorbate.

(4) Add 0.1 ml of the sodium cyanide solution to the tubes.

(5) Mix well and incubate in 37° C water bath for 4 hours. Agitate the blood suspensions every hour.

d. Interpretation. Blood deficient in G-6-PD turns brown after 1–3 hours of incubation. Normal blood remains bright red during this time.

e. Discussion:

(1) Oxalate anticoagulants cannot be used, as rapid discoloration occurs in both abnormal and normal subjects.

(2) Blood stored in ACD at 4° C can be tested for G-6-PD deficiency up to fourteen days after collection.

5-36. Shipment of Cytogenetic Specimens:

a. Guidelines:

(1) Contact the laboratory to be performing the cytogenetic studies and request media and instructions for the test. Follow explicitly the instructions of this laboratory.

(2) Send blood specimen in special media which is supplied by the Cytogenetics Laboratory. (Due to inherent instability of culture media, fresh kits are supplied only on request.)

(3) Specimens on each patient must be accompanied by Karyotype Request Form (obtained from the receiving laboratory) with brief clinical history, physical findings, and most recent white blood cell count and differential.

(4) Under normal circumstances, results of analysis are available four to six weeks after receipt of specimen.

b. Supplies Needed:

(1) 10 cc sterile disposable syringes (for example, Glaspak or Plastipak by Becton-Dickinson).

(2) Sterile needles.

(3) Heparin (Liquoemin Dosium-Organon). (If substitution is necessary, do not use heparin in which phenol is used as a preservative.)

c. Blood Collection. NOTE: It is essential that sterile conditions be maintained while drawing and transferring blood to mailing vials.

(1) Scrub the venipuncture area at least twice with 2 x 2 gauze pads soaked with 70% isopropyl alcohol (other antiseptic agents may be toxic to cultures).

(2) Allow alcohol to dry completely and, without additional palpation (which may introduce contamination) draw 8-10 cc venous blood into a sterile syringe containing 1000 units of heparin.

(3) Withdraw needle and immediately remove needle from syringe.

(4) Immediately empty syringe into blood separation vial and seal tightly.

(5) Mark vial with patient's name, time, and date specimen was drawn.

d. Mailing Instructions. Ship immediately via AIR MAIL SPECIAL DELIVERY. Specimens are usually viable for up to 72 hours after drawing. Whenever possible, therefore, specimens should not be shipped Thursday or Friday to avoid weekend arrivals.

Chapter 6

BLOOD COAGULATION

SECTION A—INTRODUCTION

6-1. Hemostasis:

a. Coagulation of the blood is only one of the components in the larger function of stopping blood flow known as hemostasis. Hemostasis is a complex process in which several factors work together or in sequence to stop the flow of blood from an injured blood vessel. The three main mechanisms involved in this process are grouped as follows: extravascular, vascular, and intravascular.

b. The extravascular mechanism involves the physical effects of the tissues (skin, muscle, etc.) which surround blood vessels. The effectiveness of this mechanism in slowing the flow of blood depends on the tissue's thickness, weight, tightness, and ability to contract. This mechanism also provides chemical factors (tissue thromboplastin) which aid in the clotting process.

c. The vascular mechanism involves the veins, arteries, and capillaries themselves. Their effectiveness depends on thickness of the vessel wall and its structure, contractibility, and retractibility.

d. The intravascular mechanism involves coagulation of the blood. This is a highly complicated mechanism that requires four stages for completion. These stages are the generation of thromboplastin, the generation of thrombin, the production of fibrin, and the dissolution of the fibrin clot.

6-2. Coagulation:

a. Blood coagulation is the formation of a clot from liquid blood. It is a complex subject and many aspects of nomenclature, physiology, and interpretation of tests remain controversial. When bleeding occurs, clotting is initiated by aggregation of platelets. The platelets congeal to plug the site of the injury. The congealing (viscous metamorphosis) process is stimulated by contact with collagen or by the formation of thrombin. Hemostasis is not achieved without the simultaneous formation of fibrin. Platelet and plasma factors are activated, and by a complex process a fibrin clot is formed. The arrest of bleeding is attained when a firm fibrin network seals the blood vessel wound with enough strength to withstand the impact of intravascular pressure.

b. Bleeding disorders occur in the following areas: injury to the vascular system, inadequate platelets, inadequate fibrin clotting mechanisms, and inadequate fibroblastic repair. The laboratory performs a variety of tests which assist the physician in his investigation of blood coagulation. Several of these tests measure the overall coagulation process. The bleeding disorders are due to one or several of the many factors involved in this process. In most instances, prolonged bleeding is due to a deficiency of one factor or another. However, in some instances it is due to therapeutic anticoagulants that are intentionally injected to interfere with the coagulation mechanism. In a few rare instances prolonged bleeding is due to a natural or antigenically-stimulated increase in circulating anticoagulants produced in the body.

SECTION B—COAGULATION FACTORS

6-3. Blood Factors. See Table 6-1 for a complete list of factors and their synonyms.

TABLE 6-1. NOMENCLATURE OF COAGULATION FACTORS

Factor Numeral	*Description Name*
I	Fibrinogen
II	Prothrombin
III	Thromboplastin
IV	Calcium
V	Labile Factor, Proaccelerin, AC-globulin
VII	Proconvertin, Stable Factor
VIII	Antihemophilic Factor (AHF) Antihemophilic Globulin (AHG)
IX	Plasma Thromboplastin Component (PTC), Christmas Factor
X	Stuart-Prower Factor
XI	Plasma Thromboplastin Antecedent (PTA)
XII	Hageman Factor, Glass Factor
XIII	Fibrin Stabilizing Factor (FSF), Fibrinase

a. Factor I (Fibrinogen). Fibrinogen, a plasma protein, is converted into fibrin in the presence of thrombin. The major source of fibrinogen is the liver. Bleeding due to a fibrinogen deficiency does not usually become manifest until the plasma concentration is below 75 mg per dl.

b. Factor II (Prothrombin). This substance is a stable glycoprotein, synthesized in the liver if an adequate amount of vitamin K is available. Prothrombin is the inactive precursor of thrombin.

c. Factor III (Thromboplastin). Thromboplastin is a variety of lipoproteins derived from platelets, the brain the lungs, and other tissues. Derived from the tissues, thromboplastin requires calcium and factors V, VII, and X to convert prothrombin to thrombin. Plasma thromboplastin (formed during the actual clotting process from factors VIII, IX, XI, XII, and platelets) requires calcium and factors V and X to convert prothrombin to thrombin.

d. Factor IV (Calcium). Calcium is an inorganic ion which is necessary for clotting to occur. Calcium functions as an activator of thromboplastic products (Stage I), a cocatalyst in the conversion of prothrombin to thrombin (Stage II) and in the formation of fibrin (Stage III). A decrease in serum calcium sufficient to interfere with flood coagulation is incompatible with life.

e. Factor V (Labile Factor Proaccelerin, Accelerator Globulin). Factor V is derived from plasma globulin, and it acts as an accelerator in the conversion of prothrombin to thrombin in the presence of tissue thromboplastin. Factor V is not present in serum because it is consumed during the clotting of blood.

f. Factor VI (Accelerin). Factor VI has been eliminated as an entity by the International Committee on Blood Clotting Factors.

g. Factor VII (Stable Factor, Proconvertin). Factor VII is stable to both heat and storage. It is thought to act as an accelerator in the conversion of prothrombin to thrombin. Factor VII is not consumed in the clotting process; therefore, it has a high concentration in serum. Severe deficiency of factor VII causes a decrease in prothrombin activity.

h. Factor VIII (Antihemophilic Factor, Antihemophilic Globulin). Factor VIII is essential to the formation of intrinsic blood thromboplastin in the first stage of clotting. Deficiency of factor VIII results in the reduction of thromboplastin as well as decreased conversion of prothrombin. Factor VIII deficiency is a hereditary sex-linked disorder transmitted by females and manifested almost exclusively in males (Hemophilia A).

i. Factor IX (Plasma Thromboplastin Component, Christmas Factor). Factor IX influences the amount of thromboplastin formed. This factor is not consumed in the clotting process; therefore, it is present in serum. Deficiency of factor IX is either hereditary or acquired and is known as Hemophilia B or Christmas disease.

j. Factor X (Stuart Prower Factor). Factor X aids in the prompt conversion of prothrombin to thrombin. Deficiency of factor X is either acquired or hereditary.

k. Factor XI (Plasma Thromboplastin

Antecedent). Factor XI aids in the formation of plasma thromboplastin. This factor is stable and is found in plasma or serum. Deficiency of factor XI is probably hereditary and results in a mild hemophilia.

l. Factor XII (Hageman Factor, Glass Factor). This factor is not required for normal hemostasis, but it is important in the various in vitro assays of the clotting mechanisms. It is a plasma contact factor with glass and is absorbed onto glass. Factor XII is related to factor XI in the activation of thromboplastin and behaves like an enzyme for which one substrate is factor XI.

m. Factor XIII (Fibrin Sabilizing Factor, Fibrinase). Factor XIII converts a loosely linked, fibrin clot (in the presence of the calcium ions) into a tough gel. Its activity is greatly reduced in serum (as compared with plasma) because of its strong adsorption of fibrin.

6-4. Platelet Factors. Platelets are active in blood coagulation. They perform the following functions: aid in vasoconstriction and the formation of a hemostatic plug, thromboplastic activity, and clot retraction. When platelets contact a wettable surface, at first they adhere to one another and then rupture, releasing chemical factors.

a. Platelet Factor 1. This factor accelerates prothrombin conversion and is actually blood factor V adsorbed on platelets.

b. Platelet Factor 2. Factor 2 accelerates fibrinogen clotting of thrombin.

c. Platelet Factor 3. A phospholipid substance involved in prothrombin activation. This is the most important factor and probably is an actual intrinsic component of platelets.

d. Platelet Factor 4. This factor reacts to neutralize heparin.

e. Platelet Factor 5. This factor is adsorbed intrinsic fibrinogen.

f. Platelet Factor 6. This factor reduces fibrinolytic activity.

g. Platelet Factor 7. This factor is adsorbed blood factor VII.

6-5. Fibrinolytic Factors. Fibrinolysis is the dissolution of a fibrin clot. The process is a necessary activity following clot formation. The mechanism of clot dissolution is complex and involves a variety of factors. In active circulating plasma profibrinolysin (plasminogen) is converted to its active form, fibrinolysin (plasmin), by tissue activators, streptokinase, urokinase, and unknown activators. Fibrinolysin acts locally to dissolve the clot.

SECTION C—THE COAGULATION MECHANISM

6-6. Introduction. The classical theory of Morowitz proposed that four components interact to form a clot as follows:

$$\text{Prothrombin} \xrightarrow[\text{Calcium}]{\text{Thromboplastin}} \text{Thrombin}$$

$$\text{Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin clot}$$

From this concept, the modern theory was devised. The modern theory is based on four stages: (1) the formation of thromboplastin, (2) the conversion of prothrombin to thrombin, (3) formation of an insoluble fibrin clot through the interaction of fibrinogen and thrombin, and (4) the lysis of the fibrin clot by fibrinolysin. These stages are illustrated in figure 6-1.

6-7. The Modern Theory:

a. Stage I. Stage I involves the formation of intrinsic (plasma) thromboplastin. This stage is initiated by the platelets adhering and rupturing, releasing platelet factor 3. Platelet factor 3 reacts with AHF (factor VIII) and PTC (factor IX) in the presence of calcium (factor IV), PTA (factor XI), factor XII, factor V, and factor X to form intrinsic thromboplastin. Tissue thromboplastin on extrinsic thromboplastin is released by the affected tissues. The average time of this reaction is 9 minutes.

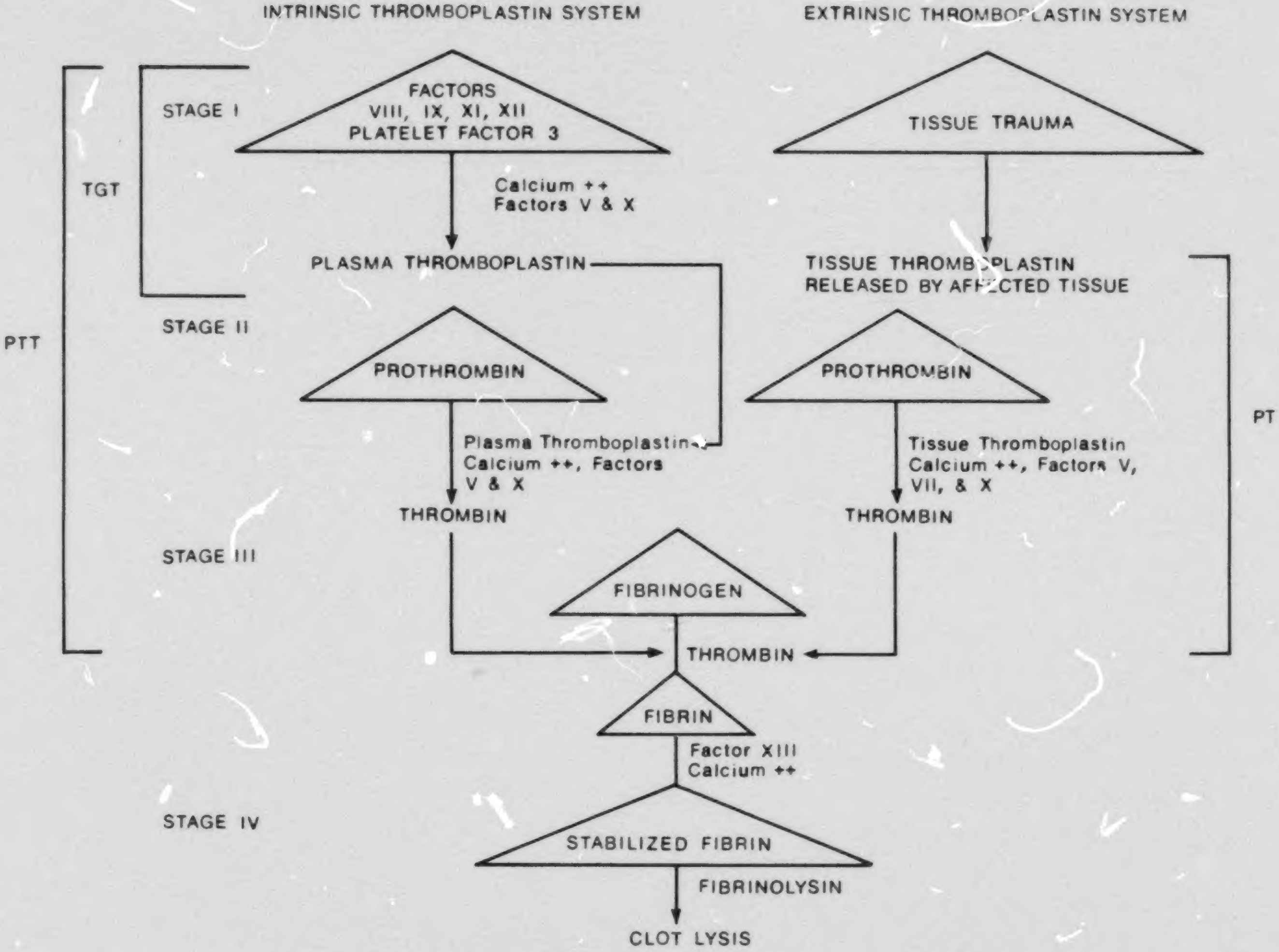

INTRINSIC THROMBOPLASTIN SYSTEM
EXTRINSIC THROMBOPLASTIN SYSTEM
PTT
TGT
STAGE I
FACTORS VIII, IX, XI, XII PLATELET FACTOR 3
TISSUE TRAUMA
Calcium ++ Factors V & X
PLASMA THROMBOPLASTIN
TISSUE THROMBOPLASTIN RELEASED BY AFFECTED TISSUE
STAGE II
PROTHROMBIN
PROTHROMBIN
PT
Plasma Thromboplastin Calcium ++, Factors V & X
Tissue Thromboplastin Calcium ++, Factors V, VII, & X
THROMBIN
THROMBIN
STAGE III
FIBRINOGEN
THROMBIN
FIBRIN
Factor XIII Calcium ++
STAGE IV
STABILIZED FIBRIN
FIBRINOLYSIN
CLOT LYSIS

Figure 6–1. Modern Theory of Coagulation.

b. Stage II. In the intrinsic system prothrombin is converted to thrombin in the presence of plasma thromboplastin, calcium, and factors V and X. The extrinsic system requires the presence of an additional factor, factor VII, for the conversion of prothrombin to thrombin. This stage takes place in 12–15 seconds.

c. Stage III. After the thrombin is generated, it quickly reacts with fibrinogen to form a fine fibrin clot. The fibrin clot plus calcium ions and factor XIII, the fibrin-stabilizing factor, form a stabilized clot. The clot is characterized by its insolubility in 5 M urea. The stage takes place in one second.

d. Stage IV. This stage is a fibrinolytic dissolution of the clot. Circulating plasminogen is converted to its active form plasmin. The clot in the presence of plasmin is dissolved.

6-8. Coagulation Inhibitors. In addition to the factors necessary for clot formation, inhibitors are present which control but do not prevent coagulation. Natural inhibitors have been described for virtually every clotting factor. Clotting activity is also inhibited by the administration of anticoagulants such as heparin and coumarin derivatives.

SECTION D—COAGULATION STUDIES

6-9. Introduction. The partial thromboplastin test is the most useful screening method for the detection of blood coagulation disorders. This test encompasses all three stages of coagulation and indicates an abnormality in all of the clotting factors, with the exception of factor VII and platelets. The prothrombin time and thromboplastin generation time detect deficiencies in other stages. The prothrombin time detects abnormalities in stages II and III while the thromboplastin generation test detects abnormalities in state I. A systematic approach should be followed in a coagulation study. Specific tests to measure the various coagulation functions are listed in Table 6-2.

TABLE 6-2. DIAGNOSTIC COAGULATION TESTS

Coagulation Function	*Test*
Vascular Function	Bleeding Time, Tourniquet Test
Platelet Function	Bleeding Time, Clot Retraction, Tourniquet Test, Prothrombin Consumption, Thromboplastin Generation Test, Platelet Count
Clotting Ability	Clotting Time, Partial Thromboplastin Time, Thrombin Time
Stage I Defects	Prothrombin Consumption Test, Thromboplastin Generation Test
Stage II Defects	One Stage Prothrombin Time
Stage III Defects	One Stage Prothrombin Time, Fibrinogen Level
Stage IV Defects	Clot Retraction, Thrombin Time, Fibrinolysis Assay

6-10. Blood Collection:

a. Two-Syringe Technique. To avoid contamination of blood by tissue juices the two-syringe venipuncture is usually employed (except for the one-stage prothrombin time) for coagulation tests using venous blood. The technique is as follows:

(1) Cleanse the site with 70% isopropyl alcohol and allow to dry.

(2) Place a tourniquet on the arm.

(3) Immediately insert the needle and remove the tourniquet. (Quick removal of the tourniquet prevents stasis.)

(4) Withdraw about 2 ml of blood. Remove the syringe and discard it.

(5) Attach a second syringe and withdraw the required quantity of blood.

(6) Blood samples for anticoagulation should be immediately mixed with the anticoagulant.

b. Anticoagulants. Anticoagulants for coagulation studies can be obtained in commercially-prepared vacuum tubes or prepared in the laboratory. The preparation and use of anticoagulants is as follows:

(1) Sodium Citrate, 3.8%. Dissolve 3.8 g of sodium citrate in 100 ml distilled water. Refrigerate. As an anticoagulant, combine one part 3.8% sodium citrate with 9 parts blood.

(2) Sodium Oxalate, 0.1 M. Dissolve 1.34 g sodium oxalate in 100 ml distilled water. Refrigerate. As an anticoagulant, combine one part 0.1 M sodium oxalate with 9 parts blood.

(3) EDTA. Dissolve 10 g of EDTA salt in 100 ml distilled water. Pipet 1 ml of this solution into a suitable test tube. Allow to dry in the oven at low temperature. For use as an anticoagulant, add 5 ml of venous blood and mix well.

c. Glassware. All glassware for coagulation studies must be scrupulously clean. Used glassware should be free of chemicals and any traces of human blood components. All glassware should be cleaned in detergents free of organic solvents and rinsed several times with distilled water. Never use test tubes or pipets that are damaged. The use of disposable syringes and needles eliminates the need for siliconizing glassware.

6-11. Bleeding Time:

a. Principle. The bleeding time is the time it takes for a small standardized wound to stop bleeding. It is dependent upon the elasticity of the skin and capillary vessels, the efficiency of tissue fluids, and the mechanical and chemical action of the platelets.

b. Duke Method:

(1) The puncture site is about 15-20 mm above the rounded fatty portion of the ear lobe.

(2) Clean the area on the ear with 70% isopropyl alcohol and allow to dry.

(3) Place a glass slide behind the ear.

(4) Puncture the thinnest part of the ear with a surgical knife blade. Start the stopwatch immediately after the puncture is made. If a good puncture is made, you will hear a click of the blade touching the glass slide.

(5) At intervals of 30 seconds, gently blot the blood from the wound with a piece of filter paper, being careful not to touch the skin.

(6) At the time when blood fails to appear on the filter paper, stop the stopwatch.

c. Ivy Method:

(1) Place a blood pressure cuff on the patient's upper arm and inflate it to 40 mm Hg.

(2) Cleanse the forearm with 70% isopropyl alcohol and allow to dry.

(3) After the area has dried, make three incisions 3 mm deep and 3 mm long with a sterile surgical blade. Avoid superficial veins.

(4) Start a stop watch and blot the three punctures gently at 15 second intervals.

(5) Continue until the bleeding stops from all wounds. Record the longest time.

d. Sources of Error:

(1) An inadequate puncture results in a poor flow of blood. Squeezing the site to obtain a free flow of blood must not be done.

(2) Low skin temperature produces a constriction of the capillary vessels resulting in decreased blood flow.

(3) A standardized puncture is necessary for valid results. Too deep a wound prolongs the bleeding time while a shallow wound shortens the bleeding time.

e. Discussion:

(1) The Ivy Bleeding Time is the method of choice because the blood pressure on the vessels is constant, the incision is uniform, and the arm offers an area for multiple determinations.

(2) The bleeding time depends primarily on extravascular and vascular factors and, to a lesser degree, on the factors of coagulation. The chief factor controlling

bleeding from a small cut is the constriction of the minute vessels following injury. Accuracy in this test is enhanced by blotting the drops of blood at shorter intervals of time as the drops of blood become progressively smaller.

(3) Thrombocytes play an important part in the formation of the hemostatic plug which seals off a wound. In thrombocytopenic purpura there is a decrease in platelets resulting in a prolonged bleeding time due to a defective platelet plug. An additional factor prolonging the bleeding time in this condition is a defect in capillary contraction.

(4) In hemophilia the bleeding time is normal. This is explained by the fact that there are no vascular or extravascular abnormalities. However, the test should not be performed on a known hemophiliac, for delayed oozing of blood is a real hazard.

f. Normal Values:

(1) Duke Method:

(a) One to 3 minutes.

(b) Three to 10 minutes borderline.

(2) Ivy Method: Up to 7 minutes.

6-12. Whole Blood Clotting Time (Lee-White):

a. Principle. The whole blood clotting time is a rough measure of all intrinsic clotting factors in the absence of tissue factors. Variations are wide and the test sensitivity is limited.

b. Procedure:

(1) Draw 5 ml of venous blood with a disposable plastic syringe, using the two-syringe method.

(2) Label 3 Kahn tubes 1, 2, and 3 and place 1 ml of blood in each tube. Set a clock for 60 minutes and start the clock as soon as the blood starts into the second syringe. A stopwatch can be used.

(3) Place the tubes in a 37° C water bath as soon as possible.

(4) After 5 minutes tilt tube 3 (the last tube filled) every 30 seconds until it clots. Then tilt tube 2 every 30 seconds until it clots. When tube 2 clots, tilt tube 1 in the same manner.

(5) Report the time it takes for the blood in the last tube to clot. If the blood does not clot within 60 minutes report the Lee-White time as greater than 60 minutes.

(6) Hold all tubes 24 hours for observation of clot retraction, clot lysis, and erythrocyte fallout.

c. Sources of Error:

(1) The following variables tend to decrease the clotting time: rough handling of the blood specimen, presence of tissue fluids (traumatic venipuncture), frequent tilting of the tube, and unclean tubes.

(2) The following variables tend to increase the clotting time: extreme increases in temperature, variation in pH, and performance of the test at room temperature.

d. Discussion:

(1) The sensitivity of the test is increased by the use of polystyrene or siliconized test tubes. The test is performed in the same manner and checked every minute after 25 minutes has elapsed.

(2) An activated clotting time is performed using celite as an activator.

(3) This test is of value primarily as it is used to follow heparin therapy. Its use as a screening procedure is limited due to its poor sensitivity.

(4) A prolonged clotting time immediately indicates impaired coagulation, but a normal clotting time does not exclude many serious clotting defects.

(5) In hemophilia there is a prolonged clotting time. This is due to a deficiency of factor VIII.

(6) The coagulation time is normal in thrombocytopenic purpura. This is explained by the fact that only a small number of thrombocytes need be present for normal coagulation to take place.

(7) When heparin is administered as a therapeutic anticoagulant, its effect may be

determined by the degree of prolongation of the coagulation time.

(8) The method of measurement of the coagulation time by the use of capillary blood drawn into a capillary tube is completely unreliable and should never be done, since the sample contains a large amount of tissue fluid. Furthermore, the volume of blood in a capillary tube is difficult to control. A capillary tube technique using venous blood has recently been described as having a high degree of accuracy.

e. Normal Values:

(1) Glass Tubes:

(a) Five to 15 minutes at 37° C.

(b) Up to 20 minutes at room temperature.

(2) Polystyrene or Siliconized Tubes: Twenty-five to 45 minutes.

(3) Activated Clotting Time: Up to 2 minutes 15 seconds.

6-13. Clot Retraction Test:

a. Principle. Whole blood is allowed to clot and as it clots it expresses serum. The clot retracts due to the action of platelets on fibrin.

b. Procedure:

(1) Withdraw 2 ml of venous blood using the two-syringe technique.

(2) Place 1 ml of blood into each of 2 glass test tubes and immediately incubate in a 37° C water bath. Inspect the tubes in 1 and 24 hours.

(3) Examine the tubes for retraction after incubation. Separation of the clot from the test tube is complete retraction (+4).

(4) At the end of 24 hours you may wish to measure the amount of expressed serum. The per cent serum expressed is calculated as follows:

$$\frac{\text{ml serum}}{\text{ml blood}} \times 100$$

c. Sources of Error:

(1) A fibrinogen deficiency causes a small clot which can be interpreted as adequate retraction even though it is inadequate.

(2) Active fibrinolysins cause the clot to dissolve. The presence of erythrocytes in the expressed serum suggests the presence of fibrinolysins.

(3) Certain anemic patients, with a low hematocrit value, show increased clot retraction due to the formation of a small clot.

d. Discussion:

(1) Poor clot retraction occurs in thrombocytopenia, qualitative platelet deficiency, and in cases of increased red cell mass.

(2) The clot retraction is normal in hemophilia since there are a normal number of platelets. However, the onset of contraction is often delayed in blood samples from hemophiliac patients.

(3) Tubes from the completed venous coagulation time can be used to perform this test.

(4) Attempts to quantitate the clot retraction test are generally unsuccessful.

e. Normal Values. A normal clot retracts from the sides and bottom of the test tube within 1 to 2 hours and expresses 45-60% of serum after 24 hours.

6-14. Tourniquet Test:

a. Principle. The fragility of capillaries is determined under increased pressure. The pressure partially obstructs the venous return from the arm and increases intracapillary pressure. The number of petechial hemorrhages reflects the degree of capillary fragility.

b. Procedure:

(1) Place a blood pressure cuff on the patient's arm and inflate it to a point midway between the systolic and diastolic pressure.

(2) Remove the cuff and wait 2 minutes.

(3) Examine a representative area (a circle about 2.5 centimeters in diameter) on the hand or arm for the presence of petechiae.

(4) Grade the number of petechiae as follows: 0–10 = 1+, 10–20 = 2+, 20–50 = 3+, and over 50 equal 4+.

c. Sources of Error:

(1) Mistaking skin blemishes for petechia increases the number. Note skin blemishes before the test.

(2) Capillary fragility varies at different sites.

(3) Maintaining the pressure too long causes false positives.

d. Discussion:

(1) The tourniquet test is a crude test to determine the ability of blood vessels to withstand trauma and should not be used as a screening test for surgery.

(2) Increased vascular fragility is sometimes found in qualitative and quantitative platelet defects, vitamin C deficiency, dietary ascorbic acid deficiency, and in the various purpuras. The term "purpura" is not specific but applies to a number of affections characterized by bleeding into tissue.

(3) The tourniquet test is most often performed by the physician.

e. Normal Values: 0–10 petechiae per 2.5 cm area.

6–15. Partial Thromboplastin Time (Activated):

a. Principle. Normal citrated plasma contains all clotting factors except calcium ions and platelets. Calcium ions and a partial thromboplastin (platelet-like substance) is added to the plasma and the clotting time recorded. An activator is added to make activation of the plasma independent of the surface of the tube.

b. Reagents:

(1) Activated Partial Thromboplastin. This reagent is available from commercial sources.

(2) Calcium Chloride, 0.025 M. Add 2.77 g calcium chloride to a 1-liter volumetric flask. Dilute to the mark with distilled water.

(3) Control Reagent. Obtain a suitable normal control from the Federal Supply Catalog or use plasma from a normal subject. Abnormal controls are also available from the Federal Supply Catalog.

c. Procedure:

(1) Combine 9 parts of freshly collected blood with one part 3.8% sodium citrate.

(2) Mix well and centrifuge for 5 minutes at 2000 rpm immediately after drawing the blood. Remove supernatant plasma and refrigerate until ready to use. Plasma must be tested within 3 hours.

(3) Place tubes of activated partial thromboplastin and 0.025 M calcium chloride in 37° C water bath. Incubate for about 5 minutes.

(4) Pipet 0.1 ml of activated partial thromboplastin into the desired number of tubes (prepare three tubes for each unknown and one for the control).

(5) Add 0.1 ml of patient's plasma or control to the tubes. Shake well and incubate for 2–4 minutes at 37° C.

(6) After the 2–4 minute incubation period, shake well; then forcibly blow 0.1 of 0.025 M calcium chloride into the test mixture and simultaneously start a stopwatch.

(7) Return the tubes to the incubator and after 30 seconds tilt the tube and observe for clot formation.

(8) At least two answers should agree within one second. Take an average for the final answer. Always report the control.

d. Sources of Error:

(1) Plasma must be prepared with care without disturbing the buffy coat.

(2) If plasma is left at room temperature, the test must be performed within 45 minutes. Otherwise plasma can be stored at 4° C for 3 hours.

(3) Clean glassware and accurate pipetting are essential for valid results.

(4) Do not use sodium oxalate as an anticoagulant. Sodium citrate is a better preservative and activates plasma faster.

(5) Tubes must be shaken thoroughly before and after incubation to insure adequate mixing.

e. Discussion:

(1) A partial thromboplastin is unable to compensate for a deficient plasma factor. A tissue thromboplastin gives a normal value in this test with a deficient factor.

(2) The activated partial thromboplastin reagent provides maximum surface exposure for the test to be valid.

(3) This test is an overall screening test for all three stages of coagulation with the exception of factor VII, calcium and platelet factor 3.

(4) The partial thromboplastin time is prolonged in all deficiencies of prothrombin and factor V, as well as deficiencies of all the plasma factors in the intrinsic system. To exclude an abnormality in the extrinsic system, a prothrombin time test should be performed on all abnormal plasma.

(5) In conjunction with the prothrombin time the following deficiencies can be determined:

PTT	*PT*	*Deficiency*
Abnormal	Normal	Stage I: factors VIII, IX, XI, XII
Abnormal	Abnormal	Fibrinogen, Prothrombin, factor V, and X
Normal	Abnormal	Factor VII

(6) A differential partial thromboplastin time is performed to detect the specific deficiency.

f. Normal Values: Less than 35.0 seconds, when using citrate anticoagulant.

6-16. One-Stage Prothrombin Time:

a. Principle. When optimal amounts of thromboplastin, calcium, and citrated plasma are mixed under carefully controlled conditions, fibrin strands will normally form within a matter of seconds. The interval during which time reaction occurs is the prothrombin time. This test measures the overall prothrombin activity of plasma.

b. Reagents:

(1) Thromboplastin. Available from the Federal Supply Catalog. Calcium added.

(2) Plasma, Human, Abnormal, Diagnostic. Available from the Federal Supply Catalog.

(3) Plasma, Human, Normal, Diagnostic. Available from the Federal Supply Catalog.

(4) Blood Collecting Tube With Sodium Citrate. Available from the Federal Supply Catalog. Contains 0.5 ml of 3.8% sodium citrate.

c. Procedure:

(1) Add exactly 4.5 ml of blood to the tube containing 0.5 of 3.8% sodium citrate.

(2) Centrifuge 5 minutes at 2,000 rpm as soon as possible. Immediately remove plasma to 12 x 75 mm test tube and refrigerate until ready to use.

(3) Prepare thromboplastin as described by package insert.

(4) Incubate the thromboplastin, patient plasma, and control plasmas for 5 minutes in a 37° C water bath. Plasma may not be incubated for more than 10 minutes, as heat destroys factor VIII.

(5) Pipet 0.2 ml of thromboplastin into the desired number of tubes.

(6) Blow in 0.1 ml of patient or control plasma, simultaneously starting a stopwatch.

(7) Incubate at 37° C for 5–6 seconds.

(8) To read: wipe the tube dry and hold toward an adequate light source. Tilt tube very gently once or twice and observe for appearance of the fibrin web which is the endpoint.

(9) Run the patient plasma and control in triplicate. The three determinations should agree as follows:

Prothrombin time	*Variation*
12-20 seconds	0.5 second
20-30 seconds	1.0 second
Over 30 seconds	2.0 second

d. Sources of Error:

(1) Timing is critical. The stopwatch must be started the instant the plasma contacts the thromboplastin, and it must be stopped immediately when the clot forms.

(2) Thromboplastin must be prepared as stated in the manufacturer's directions.

(3) Avoid traumatic venipuncture.

(4) Tourniquet application must not be prolonged.

(5) The test must be accomplished within 3 hours after collection of the blood specimen. If the test is not run immediately, refrigerate the plasma and thromboplastin. Refrigerated plasma is stable for a maximum of 4 hours.

(6) The blood must be added to the anticoagulant immediately to avoid clot formation.

(7) The test must be performed at the temperature of 37° C; incubation must not be prolonged.

(8) Clean glassware is essential. After each use, the glassware must be scrupulously cleaned with soft soap and a test tube brush and thoroughly rinsed. Synthetic detergents should not be used. Unscratched glassware reserved solely for prothrombin determinations should be utilized.

(9) The use of sodium oxalate as anticoagulant decreases the activity of factor V. Use sodium citrate as stated in the procedure.

(10) The thromboplastin reagent must be tested for constant potency. This is determined by analyzing the normal control plasma.

(11) Hemolyzed plasma must not be used.

e. Discussion:

(1) The prothrombin activity may be reported in percentage as well as in seconds by applying the patient's prothrombin time (in seconds) to a prothrombin activity curve. Curves for different normal controls are supplied by the manufacturer. If the plasma dilution curve is to be prepared, whole normal plasma must be diluted with prothrombin free plasma. Saline should never be used in making the dilutions. The percentage activity should never be calculated by dividing the normal time by the patient's prothrombin time.

(2) The prothrombin activity of a patient's plasma has important diagnostic and prognostic significance in diseases of the liver, in vitamin K deficiency, and in the use of dicumarol as a therapeutic anticoagulant.

(3) Prolongation of the one-stage prothrombin time does not measure prothrombin deficiency alone but rather indicates some failure of conversion of prothrombin to thrombin. Specifically, the test detects deficencies of factors I, II, V, VII or X. Varying reductions in any one, or combination, of these factors prolong the one-stage prothrombin time.

(4) This test is well adapted for Fibrometer use.

f. Normal Values: 12-15 seconds.

6-17. Prothrombin Consumption Time:

a. Principle. Prothrombin is partially used up in the normal clotting process. The amount of prothrombin remaining in serum indicates the adequacy of the thromboplastin complex. The time required for a clot to form when optimal amounts of thromboplastin, fibrinogen, factor V, calcium, and serum are mixed is the prothrombin consumption time.

b. Reagents:

(1) Thromboplastin. See paragraph 6-16b(1).

(2) Normal Adsorbed Plasma Reagent.

(a) Mix 9 parts of freshly collected, normal blood with 1 part 3.8% sodium citrate.

(b) Centrifuge at 2,000 rpm for 5 minutes and remove supernatant plasma.

(c) For each ml of plasma, add 100 mg of chemically pure barium sulfate (see Federal Supply Catalog).

(d) Mix well for 3 minutes and allow to stand at room temperature for an additional 2 minutes to complete adsorption.

(e) Centrifuge the material and the upper three-quarters of the plasma.

(f) Perform a prothrombin time on the adsorbed plasma. The prothrombin time should be greater than 60 seconds; if not, repeat the absorption. NOTE: Barium sulfate-adsorbed plasma is available commercially. Also available is a reagent containing fibrinogen, factor V, thromboplastin, and calcium.

c. Procedure:

(1) Draw 5 ml of blood by a nontraumatic venipuncture.

(2) Place the blood in a test tube and allow it to clot at room temperature.

(3) Place the clotted blood in a 37° C water bath for 1 hour.

(4) Centrifuge the specimen at 3,000 rpm for 3 minutes and transfer the serum to another test tube.

(5) Place the serum, thromboplastin reagent, and barium sulfate-adsorbed plasma in a 37° C water bath for 5 minutes to equilibrate to that temperature.

(6) Pipet 0.1 ml of serum, 0.1 ml of barium sulfate-adsorbed plasma, and 0.2 ml of thromboplastin reagent into a Kahn tube in that order, starting a stopwatch simultaneously.

(7) Determine the time for the clot to form as in the one-stage prothrombin time.

(8) Repeat the procedure, commencing with step 6, for two other determinations.

(9) The three determinations should agree as follows:

Time	*Variation*
Over 30 seconds	2.0 seconds
20-30 seconds	1.0 second
12-20 seconds	0.5 second

d. Sources of Error. See paragraph 6-16d.

e. Discussion:

(1) Patients having decreases or defects in the thromboplastin complex (thrombocytopenia, hemophilia, deficiency in plasma thromboplastin antecedent or component), will not use up normal amounts of prothrombin in the clotting process. The excess of residual prothrombin in the serum will result in a serum prothrombin time of less than 20 seconds.

(2) Administration of heparin will serve to shorten the prothrombin consumption time as well as prolong the "Lee-White" clotting time. Dicumarol, on the other hand, will have no effect upon the serum prothrombin time, even while prolonging the plasma prothrombin time. This indicates that dicumarol has no influence upon the thromboplastin complex.

(3) Normal results are considered valid only if the one-stage prothrombin time is normal.

(4) The test is abnormal in platelet deficiencies and deficiencies of factors VIII, IX, X, XI, and XII.

(5) It is important that the barium sulfate-adsorbed plasma be deprothrombinated sufficiently to give a prothrombin time of over 1 minute. This reagent is a source of fibrinogen and factor V, since they are used up in the formations of a clot.

f. Normal Values:

(1) Longer than 30 seconds: Normal.

(2) Twenty-30 seconds: Doubtful.

(3) Less than 20 seconds: Abnormal.

6-18. Thromboplastin Generation Time:

a. Principle. A potent thromboplastin is generated when platelets, prothrombin-free plasma, serum, and calcium are mixed. After generation of the thromboplastin, all factors necessary to produce a clot are present except for factor I and factor II. When these factors are added to a normal thromboplastin generation mixture, a clot is detected within 7–16 seconds. With an abnormal time, deficient factors are identified by substituting adsorbed patient plasma or aged patient serum.

b. Reagents:

(1) Normal Plasma Substrate. Available from the Federal Supply Catalog. This control is used as the source of factors I, II, V, VII, and X.

(2) Partial Thromboplastin (Platelet-like Substance). Available from commercial sources.

(3) Normal Adsorbed Plasma Reagent. See paragraph 6–17b(2). The adsorbed plasma is the source of factors V, VIII, XI, and XII. Dilute normal adsorbed plasma 1:5 in saline (0.85% NaCl) and allow to stand for 1 hour at 4° C, or in an ice bath before use.

(4) Normal Aged Serum. Add 2 ml of freshly-drawn blood to a clean 13 x 10° test tube and allow to clot at 37° C for 4 hours. After the incubation, centrifuge and remove the clot. Dilute the serum 1:10 with 0.85% NaCl (saline) and allow to stand for 1 hour before using.

(5) Calcium Chloride, 0.025 M. Add 0.277 g anhydrous calcium chloride to a 100-ml volumetric flask. Dilute to the mark with distilled water.

(6) Patient's Adsorbed Plasma Reagent. Prepare in the same manner as normal adsorbed plasma, substituting patient's plasma for normal plasma.

(7) Patient's Aged Serum Reagent. Prepare in the same manner as normal aged serum substituting patient's serum for normal serum.

c. Standardization of Control Reagents:

(1) Pipet 0.1 ml of 0.025 M calcium chloride into three 12 x 75 mm test tubes.

(2) Place test tubes of the following reagents in a 37° C water bath:

(a) Partial thromboplastin reagent.

(b) Normal plasma substrate.

(c) Normal adsorbed plasma.

(d) Normal aged serum.

(e) 2.0 ml of 0.025 M calcium chloride.

(f) Four tubes containing 0.1 ml of 0.025 M calcium chloride.

(3) Prepare a generation mixture by adding the following reagents to a clear 12 x 75 mm test tube:

(a) 0.2 ml partial thromboplastin reagent.

(b) 0.2 ml normal adsorbed plasma reagent.

(c) 0.2 ml normal aged serum reagent.

(d) 0.2 ml of 0.025 M calcium chloride, simultaneously starting a stopwatch or automatic timer.

(4) At 2 minutes pipet 0.1 ml of the generation mixture into the first tube containing 0.1 ml of 0.025 M calcium chloride. Immediately add 0.1 ml of normal plasma substrate to the tube, simultaneously starting a stopwatch.

(5) Check for the first formation of a clot using the tilt-tube method. (If available, substitute a Fibrometer for detection of clot formation.)

(6) Repeat steps 4–5 at 4, 6, and 8 minutes.

(7) The normal range for the control reagents is 7–16 seconds for clot formation. This range is obtained within the 2 to 8 minute generation time.

d. Test for Patient Deficiencies:

(1) Repeat the procedure for standardization of control reagents, substituting patient's aged serum for normal aged serum and patient's adsorbed plasma for normal adsorbed plasma.

(2) If there is a marked difference between the normal generation time and the patient's generation time, repeat the test, substituting normal aged serum and normal adsorbed plasma, one at a time, for the patient's reagent in the generation mixture.

e. Interpretation:

Adsorbed Plasma	*Aged Serum*	*PT*	*PTT*	*Deficient Factor*
Abnormal	Normal	Abnormal	Abnormal	V
Normal	Normal	Abnormal	Normal	VII
Abnormal	Normal	Normal	Abnormal	VIII
Normal	Abnormal	Normal	Abnormal	IX
Normal	Abnormal	Abnormal	Abnormal	X
Abnormal	Abnormal	Normal	Abnormal	XI
Abnormal	Abnormal	Normal	Abnormal	XII
Abnormal	Abnormal	Abnormal	Abnormal	Inhibitor

f. Sources of Error:

(1) All reagents must be freshly prepared each day.

(2) Tubes of plasma substrate left at room temperature are unreliable.

(3) All U.S.P. barium sulfate preparations are not standard in reaction. Factor IX at times may be incompletely removed.

(4) Antithromboplastin activity in blood prolongs TGT, and accounts for some double deficiencies.

(5) This is not a routine laboratory procedure; considerable technologist skill is required.

(6) A clot can form when the generation mixture is prepared. This is removed with a wooden applicator stick.

(7) Other sources of error can be found in paragraphs 6-15d and 6-16d.

g. Discussion:

(1) A platelet deficiency can be detected by the TGT. This is done by preparing platelet-rich plasma and substituting it for the partial thromboplastin reagent. An abnormal generation time is noted in a patient with platelet deficiency.

(2) The TGT reveals abnormalities essential to the development of thromboplastin activity in the intrinsic blood system.

(3) The TGT should be correlated with the PT and PTT to determine the specific abnormality.

(4) Adsorbed plasma contains factors V, VIII, XI, and XII. If the TGT is prolonged when patient's adsorbed plasma is substituted for normal adsorbed plasma, a deficiency in one or more of these factors is indicated.

(5) Aged serum contains factors IX, X, XI, and XII. If the TGT is prolonged when patient's aged serum is substituted for normal aged serum, a deficiency in one or more of these factors is indicated.

(6) Factors XI and XII are present in adsorbed plasma and aged serum. A deficiency in either of these factors prolongs the TGT. Determination of the specific factor deficiency is done by the physician on the basis of clinical grounds.

h. Normal Values. Clot formation time of 7-16 seconds within 8 minutes generation time.

6-19. Thrombin Time:

a. Principle. A known amount of thrombin is added to plasma and the time required for clot formation is recorded.

b. Reagents:

(1) Thrombin, 5000 Units per ml. Available from the Federal Supply Catalog.

(2) Stock Thrombin Solution, 500 Units per ml. Reconstitute the thrombin with 10 ml of saline (0.85 percent NaCl).

(3) Working Thrombin Solution, 10 Units per ml. Dilute the stock solution 1:50 with saline (0.85 percent NaCl).

(4) Control Plasma. Available Federal Supply Catalog.

c. Procedure:

(1) Obtain 4.5 ml of venous blood and add to 0.5 ml sodium citrate. Mix well.

(2) Centrifuge and separate the plasma.

(3) Incubate patient plasma, control plasma, and thrombin solution in 37° C water bath for 3 minutes.

(4) Pipet 0.1 ml of control plasma and 0.1 ml of thrombin solution into a clean test tube, simultaneously starting a stopwatch. Observe for clot formation.

(5) Repeat step (4) for the patient's plasma.

d. Sources of Error. See paragraph 6-16d.

e. Discussion:

(1) The thrombin time is prolonged in fibrinogen levels below 100 mg per dl, presence of fibrinolysins, and presence of circulating anticoagulants.

(2) Low concentrations of heparin in the patient's plasma may not be observed by this test. To detect small amounts of heparin, thrombin is diluted, and normal and patient's plasmas thrombin times are determined. A prolongation of the patient's thrombin time over that of the normal at some dilution indicates an antithrombic substance.

(3) To perform the antithrombin test, dilute the working thrombin 1:2, 1:4, 1:8, 1:16, and 1:32 with saline. Then follow the procedure for the thrombin time test using the dilutions.

f. Normal Values: 11-15 seconds.

6-20. Fibrinogen Assay (Semiquantitative):

a. Principle. Fibrinogen, a plasma globulin formed in the liver, is salted out by ammonium sulfate and measured with a spectrophotometer.

b. Reagents:

(1) Parfentjev Reagent. Add 133.33 g ammonium sulfate, 10.0 g NaCl, and 0.025 g merthiolate to a 1-liter volumetric flask. Dilute to the mark with distilled water.

(2) Saline (0.85 Percent NaCl). Add 8.5 g sodium chloride to a 1-liter volumetric flask. Dilute to the mark with distilled water.

(3) Fibrinogen, U.S.P. Available from the Federal Supply Catalog.

(4) Fibrinogen Stock Standard, 500 mg per dl. Add 500 mg of fibrinogen to a 100-ml volumetric flask. Dilute to the mark with saline.

c. Calibration Curve:

(1) Prepare the following dilutions of the stock standard:

Fibrogen Stock Standard	*Saline*	*Concentration*
10.0 ml	0	500 mg per dl
7.5 ml	2.5 ml	350 mg per dl
5.0 ml	5.0 ml	250 mg per dl
2.5 ml	7.5 ml	125 mg per dl

(2) Set up the following cuvets for each standard:

Blank Cuvet	*Test Cuvet*
0.5 ml standard	0.5 ml standard
4.5 ml saline	4.5 Parfentjev reagent

(3) Three minutes after addition of the Parfentjev reagent, shake the cuvets vigorously, and read the absorbances of the test cuvets at 510 nm with the blank set at zero absorbance.

(4) Plot absorbance versus concentration on linear graph paper.

d. Procedure:

(1) Draw 4.5 ml of fresh venous blood and add to a test tube containing 0.5 ml of 3.8% sodium citrate.

(2) Centrifuge and separate the plasma.

(3) Set up the following cuvets for each unknown plasma:

Blank	*Unknown*
0.5 ml plasma	0.5 ml plasma
4.5 saline	4.5 Parfentjev reagent

(4) Three minutes after addition of the Parfentjev reagent, shake the cuvets vigorously, and read the absorbances of the test cuvets at 510 nm with the blank set at zero absorbance.

(5) Obtain the fibrinogen concentration from the calibration curve.

e. Sources of Error:

(1) The Parfentjev reagent deteriorates after two weeks of storage. Prepare fresh every 2 weeks.

(2) The fibrinogen has a potency for 60 months. Do not use outdated fibrinogen to prepare standards.

(3) The procedure is limited as it is dependent on time, temperature, anticoagulant, and concentration of other proteins.

f. Discussion:

(1) Although the procedure is limited, a rapid determination can be obtained when adequate fibrinogen levels are present.

(2) Screening procedures, in kit form, are available commercially.

(3) Fibrinogen is essential to clot formation in stage 3 of the clotting mechanism. Bleeding is encountered when the plasma fibriogen level falls below 75 mg per dl.

(4) A colorimeteric fibrinogen procedure is outlined in AFM 160-49.

g. Normal Values: 200-400 mg per dl.

6-21. Plasma Recalcification Time:

a. Principle. Calcium chloride is added to plasma and the clotting time recorded.

b. Reagents:

(1) Calcium Chloride, 0.025 M. See paragraph 6-18b(5).

(2) Sodium Citrate, 3.8 Percent. Available from the Federal Supply Catalog.

c. Procedure:

(1) Draw 4.5 ml of fresh venous blood and add to 0.5 ml of 3.8% sodium citrate.

(2) Centrifuge at 2,500 rpm for 20 minutes and separate the plasma.

(3) Add 0.2 ml of patient's plasma and 0.2 ml of normal control plasma to two test tubes in a 37° C water bath.

(4) Add 0.2 ml of 0.025 M calcium chloride to each tube, simultaneously starting a stopwatch.

(5) Tilt the tube every 30 seconds for the first 90 seconds. Remove the tube and observe for clot formation.

d. Sources of Error:

(1) Platelet-poor plasma must be used in the test. Platelet-rich plasma shortens the recalcification time.

(2) The test is dependent on the platelet count, concentration of plasma clotting factors, time of storage in glass, and the presence of circulating anticoagulants.

(3) See paragraph 6-15d for other sources of error.

e. Discussion:

(1) This test is the basis for other coagulation procedures.

(2) An abnormal result occurs in deficiencies of factors VIII, IX, X, fibrinogen, and the presence of a circulating anticoagulant.

f. Normal Values: 90-120 seconds.

6-22. Detection of a Circulating Anticoagulant:

a. Principle. An abnormal recalcification time is not corrected by the addition of normal plasma if a circulating anticoagulant is present.

b. Reagents. See paragraph 6-21b.

c. Procedure:

(1) Obtain citrated plasma from a normal donor and from the patient.

(2) Set up the following mixture in 12 x 75 mm test tubes:

Tube No.	Patient's Plasma	Normal Plasma	% Patient's Plasma
1	0.20 ml	0	100%
2	0.15 ml	0.05 ml	75%
3	0.10 ml	0.10 ml	50%
4	0.05 ml	0.15 ml	25%
5	0.02 ml	0.18 ml	10%
6	0	0.20 ml	Control

(3) Add 0.2 ml of 0.025 M $CaCl_2$ to each tube and determine the plasma recalcification time (see 6–21) on each tube.

d. Interpretation:

(1) The abnormal recalcification time is corrected by normal plasma if no circulating anticoagulant is present.

(2) A circulating anticoagulant present in patient's plasma prolongs the recalcification time of normal plasma.

6–23. Detection of Factor XIII Deficiency:

a. Principle. Factor XIII converts a loosely-linked, fibrin clot (in the presence of calcium ions) into a tough gel. The well-formed clot is insoluble in 5 M urea. In the absence of factor XIII, the clot lyses within 3 hours

b. Reagents:

(1) Sodium Citrate, 3.8%. Available from the Federal Supply Catalog.

(2) Calcium Chloride, 0.025 M. See paragraph 6–18b(5).

(3) Urea Solution, 5 M. Add 30 g of urea to a 100-ml volumetric flask. Dilute to the mark with distilled water.

c. Procedure:

(1) Draw 4.5 ml of fresh venous blood and add to 0.5 ml of 3.8% sodium citrate.

(2) Centrifuge at 2,500 rpm for 5 minutes and separate plasma.

(3) Pipet 0.1 ml of plasma and 0.1 of 0.025 M calcium chloride to a clean 13 x 100 mm test tube.

(4) Incubate the clot for 30 minutes in a 37° C water bath.

(5) After the incubation, loosen the clot and add 3 ml urea solution.

(6) Observe the clot for lyses at 1-hour intervals.

d. Interpretation. A clot that lyses within 3 hours is deficient in factor XIII.

6–24. Platelet Count (Rees-Ecker):

a. Principle. A sample of bood is diluted in an isotonic anticoagulant. The anticoagulant employed contains a stain and serves the dual purpose of being both a fixing diluent and a staining medium. After mixing, the specimen is introduced into the counting chamber and the number of thrombocytes in a known volume is counted.

b. Reagent. Rees-Ecker Diluting Fluid. Add 3.8 g sodium citrate, 0.2 ml 40% formaldehyde, 0.1 g brilliant cresyl blue to a 100-ml volumetric flask. Dilute to the mark with distilled water. Filter the solution just prior to use. Keep in a glass-stoppered bottle in the refrigerator. Prepare the solution frequently since in old solutions the formaldehyde oxidizes to formic acid, hemolyzing the red cells.

c. Procedure:

(1) Fill a red cell diluting pipet with freshly-filtered Rees-Ecker solution and expel it, leaving a film of the solution inside the pipet.

(2) Draw blood to the 0.5 mark in the red cell pipet.

(3) Wipe the excess blood from the outside of the pipet and dilute to the 101 mark with Rees-Ecker diluting fluid.

(4) Shake the pipet for several minutes. Discharge a few drops from the pipet. Then charge both chambers of a hemacytometer.

(5) Allow the thrombocytes to settle for 15 minutes. To prevent drying, the counting chamber may be placed on a damp towel and covered with a Petri dish cover.

(6) Under highpower magnification, count all of the thrombocytes seen in the

center 1-sq-mm area of each of the two charged chambers of the hemacytometer. With proper adjustment of the light and continual fine focusing, the thrombocytes are seen as blue, highly-refractile bodies, which may be round, oval, or comma-shaped. They vary in size from one-five microns.

d. Calculations. The volume in both chambers is 0.2 cu mm (area x depth = 2 sq mm x 0.1 mm = 0.2 cu mm). The formula for calculation of indirect platelet count is as follows:

$$\frac{\text{Total platelets counted} \times \text{dilution}}{\text{volume}} = \text{platelets per cu mm}$$

or

$$\frac{\text{Total platelets counted} \times 200}{0.2} = \text{platelets per cu mm}$$

e. Sources of Error:

(1) Platelet agglutinates invalidate the count. This is prevented by using scrupulously clean glassware. Therefore, the following precautions are recommended:

(a) Boil counting chambers and coverslips for 2 hours in distilled water to which a small amount of sodium bicarbonate has been added.

(b) Pipets must be very carefully cleaned.

(c) The diluting fluid must be frequently filtered or centrifuged and stored at 2° to 4° C.

(2) Other sources of error as listed under red and white blood cells counts also apply, including pipetting errors. These procedures should be consulted.

(3) More accuracy may be obtained through use of the phase contrast microscope.

f. Discussion:

(1) No one method for the enumeration of thrombocytes is satisfactory in every respect. In experienced hands the direct procedure is more accurate than the indirect method, and venous blood samples are more representative and preferable to those obtained by capillary puncture. Direct procedures, however, require greater skill and experience for proper performance and interpretation. The error in a single count has been estimated to be about 10 percent, and this margin of error can be reduced by multiple counts.

(2) A major disadvantage of the direct thrombocyte count is that the platelets must be counted under high-power rather than oil-immersion magnification as is used in the indirect method. Because of the extremely small size of blood platelets, it is a common error to confuse them with yeasts, debris, and precipated stain.

(3) Due to the inherent errors in this procedure, it is recommended that a thrombocyte count be performed on a normal person as a control.

g. Normal Values: 150,000-350,000 platelets per cu mm. NOTE: This range is generally acceptable, but it must be realized that there is a great variance in normal values which differ with each technique, laboratory, and technician.

6-25. Platelet Count (Phase):

a. Principle. Blood is diluted with ammonium oxalate, and the diluted specimen is introduced into a counting chamber. The platelets appear round or oval, pink, purple, or even black under a phase condenser.

b. Reagent: Ammonium oxalate, 1%. Add 1 g of ammonium oxalate to a 100-ml volumetric flask. Dilute to the mark with distilled water. Store in the refrigerator and filter before use.

c. Procedure:

(1) Draw 5 ml of venous blood and immediately place in a test tube containing EDTA. (If capillary blood is used, immediately fill a red blood cell diluting pipet to the 1 mark.)

(2) Fill a red blood cell diluting pipet to the 1 mark and then draw ammonium oxalate to the 101 mark.

(3) Shake the pipet 3 to 5 minutes.

(4) Discard from 1/3 to 1/2 the volume from an RBC pipet, then charge a special flat bottom-phase counting chamber. Place the counting chamber in a Petri dish containing moist gauze and let stand 15 to 20 minutes to insure complete settling of platelets.

(5) Count the platelets in all 25 squares of the area normally used for the red cell count. Multiply the results by 1,000.

(6) Both sides of each chamber are filled with the same sample; the count from either side should not deviate more than 10% from the other count. If a greater than 10% deviation occurs, repeat the count using a fresh dilution and a second chamber.

(7) Report the average of the two sides counted.

d. Sources of Error:

(1) The diluting fluid must be fresh and free of bacterial contamination.

(2) Platelet clumping occurs if there is delay in adding blood to the anticoagulant or if the mixing is inadequate. Platelet clumps cause invalid results.

(3) Occasionally extraneous material is mistaken for platelets.

(4) Glassware that is not scrupulously clean causes platelets to attach to the debris on the glassware.

(5) See paragraph 6-24e for other sources of error.

e. Discussion:

(1) Platelets sometimes show dendritic processes. Structures such as dirt, crystals, and WBCs are refractile. Platelets are not. The white cells are normally lysed; however, with patients that demonstrate excessive high white counts some cells will be observed in the platelet counting prep.

(2) Ammonium oxalate insures clearing of the background by hemolysis.

(3) This method is more accurate than the Rees-Ecker. The main drawback to this method is that special equipment is required.

(4) See paragraphs 6-24f for further discussion.

f. Normal Values: 150,000 to 350,000 per cu mm.

6-26. Macroglobulin Distilled Water Screening Test:

a. Principle. Macroglobulins are precipitated when brought into contact with distilled water.

b. Procedure:

(1) Collect 5 ml of blood from the patient by venipuncture.

(2) Set the blood aside for 1 hour to clot, and then separate the serum by centrifuging at 2,000 rpm for 5 minutes. NOTE: Oxalated, citrated, or heparinized plasma is also satisfactory for the performance of this test.

(3) Place 5 ml of distilled water in a Wassermann tube.

(4) With a 1 ml pipet draw up 0.5 ml of patient's serum or plasma. Holding the pipet tip close to the meniscus of the water, add 0.2 ml of the patient's serum or plasma to the distilled water.

(5) Interpret results.

c. Interpretation:

(1) Observe the behavior of the drop of serum or plasma sinking in the water and the modifications of color of the solution.

(2) If macroglobins are present:

(a) The drop will sink leaving a smoky trace.

(b) The entire distilled water medium will promptly assume a whitish color, due to the condensation of transparent, slimy masses forming a precipitate which quickly sinks to the bottom.

(3) The separated precipitate will dis-

solve well when transferred to a test tube containing normal saline.

6-27. Cryoglobulin Screening Test:

a. Principle. Cryoglobulins in serum or plasma are precipitated at 4° C and dissolved by temperature elevation to 37° C.

b. Procedure:

(1) Collect 10 ml of blood by venipuncture and place a 5 ml portion in a tube containing anticoagulant (citrate, oxalate, or heparin), and the other 5 ml portion into a clean sterile test tube.

(2) Incubate both tubes immediately for 1 hour at 37° C.

(3) One hour later, separate the supernatant plasma from the tube containing the anticoagulant and the serum from the clot in the untreated tube.

(4) Transfer aliquots of 1 ml of serum into two clean sterile Wassermann tubes.

(5) Transfer aliquots of 1 ml of plasma into two clean sterile Wassermann tubes.

(6) Incubate one serum specimen and one plasma specimen at 4° C (refrigerator temperature) for 4 hours.

(7) Incubate one serum specimen and one plasma specimen at 37° C for 4 hours.

(8) Read results.

c. Interpretation:

(1) The test is positive if the serum or plasma incubated at 37° C shows no changes and the serum or plasma incubated at 4° C shows the following changes:

(a) The serum or plasma appear clotted in toto.

(b) The plasma or serum appear divided into two layers; the upper one containing normal plasma or serum, faintly stained by bilirubin; the lower one represented by the cryoglobulin, whitish in color, which has precipitated to the bottom of the test tube.

(2) The test is confirmed if the serum or plasma incubated at 4° C is brought back to a temperature of 37° C and its appearance returns to normal.

Chapter 7

QUALITY CONTROL

7-1. Introduction:

a. Quality control is a means to insure the reliability of the tests performed. Unreliable results mislead the physician and could quite possibly harm the patient. Quality control can tell the laboratory the degree of uncertainty which always goes with a result. The object of a good quality control program is to keep this degree of uncertainty within narrow and usable limits. The clinical laboratory must adapt certain statistical procedures which aid in detecting inaccurate results. All data submitted by the laboratory should be substantiated by sufficient statistics to indicate that it is within reliable limits. There is no substitute for statistical validation of a result.

b. Quality control in hematology has three objectives: accuracy of results, precision of results, and detection of random errors. These objectives are accomplished in two ways: (1) By careful, diligent, and intelligent performance with due recognition of the limitations and sources of error of each procedure; (2) By statistical evaluation of results and the measures necessary to keep specimens "in control." The measures necessary involve instrument checks, technician performance, choice of procedure, etc.

7-2. Definition of Terms. The science of statistics deals with the study of data and provides the basis for application and interpretation of a quality control program. Statistics, like all other sciences, has evolved certain terms and expressions which require definition and understanding to get maximum use of this valuable tool.

a. Accuracy: The degree of approximation of a result to the actual value, or, simply put, the closeness of the observed value to the actual value. Accuracy of a result is dependent on: (1) preparation of patient, (2) technique used for collection of the blood specimen, (3) interval of time between collection and testing of the specimen, (4) standardized and controlled reagents and equipment, and (5) elimination of random errors.

b. Precision: The closeness with which a series of replicate measurements agree with each other. Precision is achieved when two or more replicate measurements fall within the range of allowable variation.

c. Arithmetic Mean: A simple "average." The arithmetic mean of a series of test results is obtained by averaging the test results.

d. Standard Deviation (S.D.): The range of dispersion (distribution) of values about their mean. S.D. is a descriptive tool which condenses the frequency distribution of a series of values into a single measurement unit. If a sufficiently large number of similar determinations is performed on a single sample and a distribution curve is plotted of the frequency of test results, the curve ideally forms the classical Gaussian (bell-shaped) curve found in figure 7-1. The peak of the curve is at or near the average (mean of all the test results plotted). Algebraically, standard deviation is the square root of the sum of the squared difference from the mean value divided by the total number of values less 1. It is usually expressed as

$$\text{S.D.} = \sqrt{\frac{\Sigma d^2}{n-1}}$$

where:

d = difference between each value and the mean value

Σd^2 = sum of the squared difference from the average

n = the number of determination performed. (N-1 is used only when $N<30$).

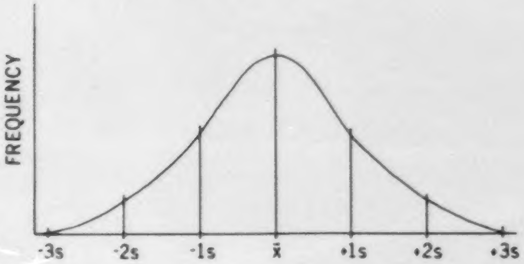


Figure 7-1. Classical Gaussian (Bell-shaped) Curve.

e. Confidence Limits or Range of Allowable Variation. The limits of a procedure are:

(1) ± 1 mean standard deviation includes 68% of all test results.

(2) ± 2 mean standard deviation includes 95% of all test results.

(3) ± 3 mean standard deviation includes 99.7% of all test results.

f. Median: That value which divides a distribution so that an equal number of values are on either side of it. For example, with values such as 6,200, 6,400, 7,000, 7,400, and 7,200, 7,000 is the median.

g. Mode: The most frequently occurring value found directly beneath the peak of a distribution (curve). It is not necessarily synonymous with median.

h. Coefficient of Variation (C.V.): This is an expression of the variation in percent rather than an absolute value. Algebraically, it is expressed as:

$$\text{C.V.} = \frac{\text{S.D.}}{\text{mean}} \times 100$$

i. Allowable Percent Variation: This is an expression of the limits within which a value must fall, in percent. Algebraically, it is expressed as:

Allowable percent variation = C.V. × confidence limits

j. Reproducibility: A measure of how much a test result varies from established mean values if the test is carried out in different laboratories by different personnel using similar equipment and procedure. It also measures variation produced when a test is performed within a laboratory by different technicians. The survey programs you participate in provide a method of determining the reproducibility of a test procedure.

k. Reliability: Proven consistency in obtaining accurate and precise results. Reliability is established by checking the test procedure at frequent intervals with control specimens. In doing so, not only is the reliability of the test procedure established but also that of the reagents and the equipment used.

l. Randomization: The avoidance of a fixed pattern in the placement of control tests within a system.

m. Random Errors: Errors which occur without direction or rule. These errors are usually due to instrument malfunction or personal factors.

n. Validity: Test results are valid if they are a measure of what was intended and are accurate, precise, and reproducible.

7-3. Statistical Evaluation of Hematology:

a. General Considerations. It must be acknowledged that exact reproduction of tests is not attainable. Variation of results is a fact. The ultimate attainment of quality control in the laboratory is to understand this variation so that confidence limits can be established, erroneous results detected, and appropriate corrective action initiated to correct any significant discrepancy. An adequate quality control system indicates the confidence of the test, and provides ample warning when a procedure is "out of control." The approach to control or statistical evaluation of hematology is to employ the standard deviation as a tool.

b. Calculation of Standard Deviation.

(1) Reconstitute the control according to instructions. NOTE. Commercial controls for red blood cell counts, white blood cell counts, coagulation studies, hemoglobin determinations, and hematocrit determinations are available. A hemoglobin control solution can be prepared using outdated blood bank blood. The method is included in this chapter.

(2) Mix the sample thoroughly by repeated inversion.

(3) Perform the desired analysis on the control.

(4) Continue analyzing the control daily for an overall total of at least 30 consecutive days.

(5) Compute the average value and standard deviation for each control measured, as shown in figure 7-2.

(a) In column A, record values obtained from daily analyses of the control.

(b) Total the values and record in block B.

(c) Determine the average of column A by dividing the total (block B) by 30 or the number of times the control was analyzed. Record the average (block C).

(d) Compute and record in column D the individual differences of each test result from the average (block C).

(e) Square each individual difference and record in column E.

(f) Total column E and record in block F.

(g) Calculate the standard deviation, as shown in block G. Consult a table of square roots in determining this value.

(h) Calculate the acceptable range for the control based ± 2 standard deviations (block H). Some laboratories may prefer to convert this to the coefficient of variation.

(i) Record the control limits for the test procedure under block I. The upper limit is determined by adding two standard deviations to the average value. The lower limit is determined by subtracting two standard deviations to the average value.

c. Preparation of Quality Control Charts. When control limits for various hematology procedures have been established, prepare quality control charts reflecting the ± 2 standard deviation range for each test, as shown in steps (1) through (5).

(1) On the left border of a sheet of graph paper, prepare a vertical scale of appropriate test unit values. The scale should extend symmetrically above and below the average value, adequately encompassing ± 2 standard deviation values. (See example, figure 7-3.)

(2) Draw a solid black line extending from the average value completely across the chart. Label the line "average" at the right border.

(3) Similarly extend lines from values representing ± 2 standard deviations (S.D.) in parallel to the average line. Appropriately label these lines ± 2 S.D.

(4) Across the bottom of the graph, record the days of the month, using two squares for each day.

(5) Label the chart with the name of the analysis, control lot number, and the month for which the control is to be used in monitoring the test.

d. Quality Control Application:

(1) On the morning of each duty day allow a control to warm up to room temperature.

(2) After thorough mixing, analyze a control sample, along with one or more clinical specimens, with each applicable procedure. Both the control sample and patient specimen must be processed by the same technician under the same conditions for any given procedure.

(3) If the control value falls within ± 2 standard deviations (S.D.), report the results of patient specimen analysis.

(4) Record the control sample result

PROCEDURE:	DATE:	CONTROL POOL NO.:	TECHNICIAN(S):
HEMOGLOBIN	3APR72	4	LT. JONES

A. Test Results	D. Differences From Average	E. Squared Diff. From Average
1. 14.1	.13	.0169
2. 14.3	.07	.0049
3. 14.3	.07	.0049
4. 14.4	.17	.0289
5. 14.1	.13	.0169
6. 14.0	.23	.0529
7. 14.1	.13	.0169
8. 14.3	.07	.0049
9. 14.1	.13	.0169
10. 14.4	.17	.0289
11. 14.4	.17	.0289
12. 14.2	.03	.0009
13. 14.3	.07	.0049
14. 14.1	.13	.0169
15. 14.1	.13	.0169
16. 14.2	.03	.0009
17. 14.2	.03	.0009
18. 14.4	.17	.0289
19. 14.4	.17	.0289
20. 14.1	.13	.0169
21. 14.2	.03	.0009
22. 14.2	.03	.0009
23. 14.1	.13	.0169
24. 14.2	.03	.0009
25. 14.3	.07	.0049
26. 14.3	.07	.0049
27. 14.2	.03	.0009
28. 14.4	.17	.0289
29. 14.2	.03	.0009
30. 14.4	.17	.0289
31.		

B. Sum of Results: 427.0

C. Average Result: 14.23

F. Sum of Squared Differences From Average: 0.4270

G. Calculation of Standard Deviation (SD):

$$SD = \sqrt{\frac{\text{Sum of Squared Differences From Average}}{\text{No. Tests}}}$$

(N-1, IF < 30)

$$SD = \sqrt{\frac{0.4270}{30}}$$

$$SD = \sqrt{0.0142}\ ^{*}$$

SD = 0.12

H. Calculation of Acceptable SD Range:

Acceptable limits = ± 2 X SD
or: 2 X 0.12 = 0.24

I. Test Control Limits

Average result: 14.2 g per dl
Upper limit: 14.4 g per dl
Lower limit: 14.0 g per dl

* Consult a table of square roots

Figure 7-2. Example of a Monthly ± 2 Standard Deviation Calculation.

on the appropriate quality control chart for the day concerned, as shown in figure 7-3.

(5) If the control value is outside the ± 2 limits, recheck test calculations. If this is not the cause, repeat the procedure on all specimens, including a new control sample. If the control sample falls within ± 2 S.D. on the second run and clinical specimen values remain largely unchanged, this probably indicates improper handling of the control sample during the first run.

(6) If the second control analysis is out of limit, a basic flaw in the test should be suspected. Carefully check for possible causes such as deteriorated reagents, improper pH, inaccurate temperature, defective instrumentation, etc.

(7) Report clinical specimen results

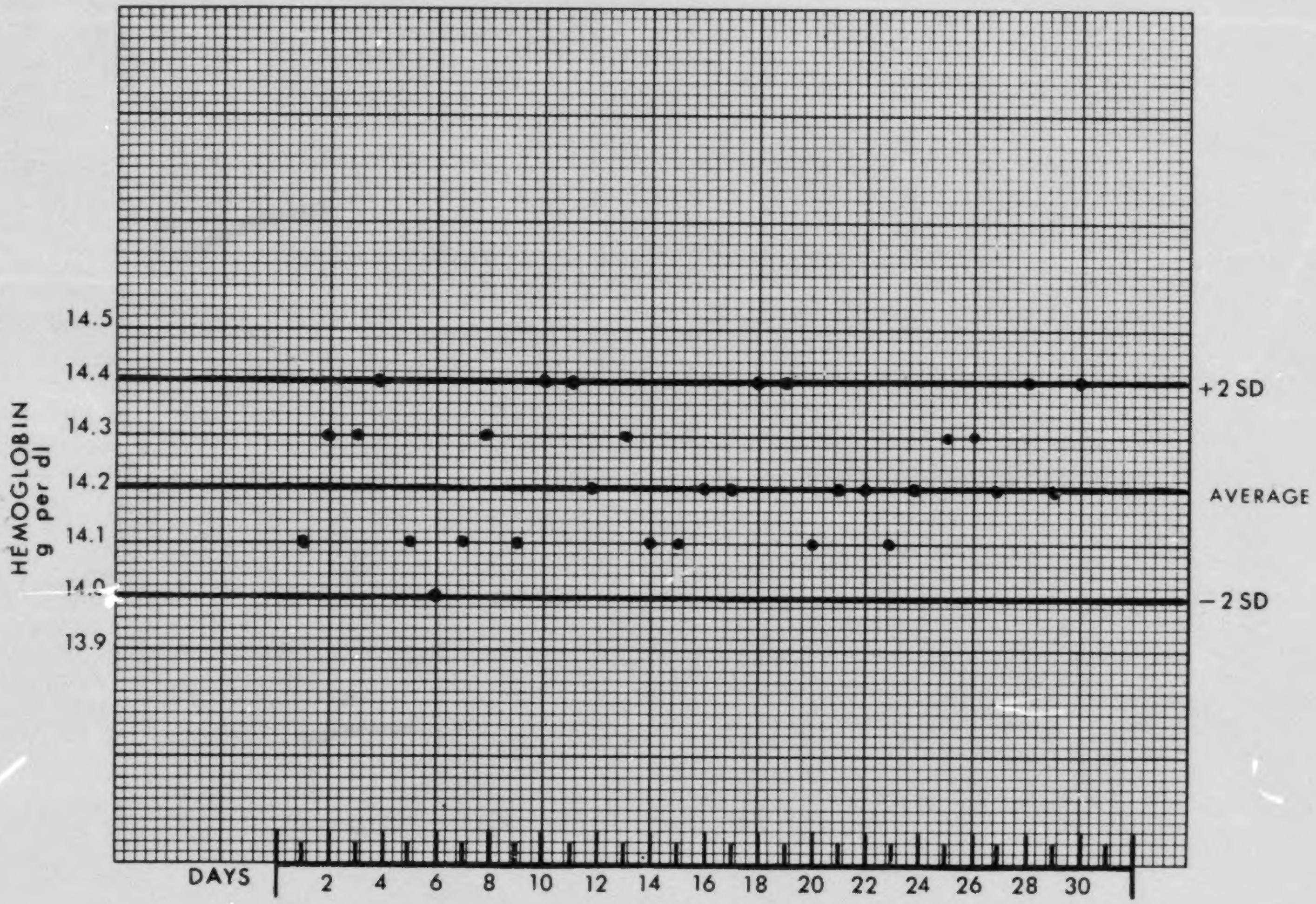


Figure 7–3. Example of a Quality Control Chart.

obtained with an accurate control reading after correction of the test discrepancy. Occasionally this may necessitate re-collection of specimens from patients.

(8) Keep a permanent record of all control sample values falling outside ± 2 S.D. in the day-to-day performance of procedures. Enter the cause, if determined, and corrective action taken for each procedure concerned. This data may prove useful in troubleshooting future analytic problems. Record all "out-of-limit" results on quality control charts since these values should be used in calculating monthly standard deviations as described below.

(9) When 1 month has elapsed, inspect the quality control charts (figure 7-3) for the various methods. Calculate the monthly standard deviation for each procedure that exhibited more or less equal distribution of control values above and below the mean. This is accomplished using the chart values accumulated during the month's run.

(10) Prepare new quality control charts reflecting the adjusted ± 2 S.D. range computed in step (9).

(11) If monthly result trends of any procedure indicate consistent progression toward one control limit or the other, restudy the method. Following any corrections in technique, reagents, or instrumentation, redetermine the ± 2 S.D. limits on a new series of 30 daily control analyses.

(12) Continue analyzing the control in conjunction with clinical specimens, and record control results on the new quality control charts serving the new month.

(13) In the manner described above, prepare quality control charts for each successive month, always using ± 2 S.D. ranges computed from control sample values of the previous month. In actual practice, this should progressively narrow the acceptable range of analytic error for each procedure.

7-4. Control in Blood Collection. The method by which a blood specimen is collected can introduce considerable variation in results. Studies have shown that massaging the finger or forcing blood from an inadequate puncture significantly affects both red and white count. The blood must be immediately placed in the desired anticoagulant and mixed well. Differential slides should be prepared from fresh blood or from EDTA anticoagulated blood very soon after collection. All specimen containers must be properly identified with the patient's name. Proper collection of blood specimens prevents one source of random errors.

7-5. Control in Hemoglobin Determinations:

a. Preparation of Control Solutions:

(1) Centrifuge at least 20 ml of anticoagulated blood having a high hemoglobin content. Remove the plasma.

(2) Wash the cells 3 times in 0.9% NaCl (physiologic saline), removing as much saline as possible following the last centrifugation.

(3) Freeze the washed cells overnight to obtain hemolysis. If complete hemolysis is not obtained, refreeze for a second night.

(4) To the thawed hemolyzed cells add 1/2 volume of chloroform and shake vigorously for 1 minute.

(5) Centrifuge immediately for 15 minutes at 3,000 rpm.

(6) Remove the supernatant hemoglobin solution to graduated tubes and record the volume.

(7) Analyze the solution for hemoglobin concentration in g per dl.

(8) Adjust the hemoglobin concentration to approximately 14.0 g per dl with glycerin. The glycerin is also a preservative for the solution. The volume of glycerin added is calculated as follows:

$$\text{ml glycerin} = \frac{\text{actual concentration}}{14.0} - 1 \times \text{volume of control solution}$$

(9) Mix thoroughly after addition of glycerin. Store in the refrigerator. This so-

lution is stable up to 10 months in the refrigerator.

(10) Use as whole blood to verify routine hemoglobin determinations.

b. Calibration:

(1) Sahli Pipets. The procedure for calibration of Sahli pipets is outlined in paragraph 2-6. At least 3 pipets should be calibrated to the nearest 0.02 ml. These are reference pipets. All new pipets can be compared colorimetrically with the reference pipets. An accurate pipet must produce a hemoglobin solution in Drabkin's identical to solutions prepared from reference pipets. The allowable error is ± 0.5% T.

(2) Dilutors or Diluting Pipets. These instruments are checked with 5 ml volumetric flasks. The volume must be 5 ml. Duplicator hemoglobin solutions prepared from these instruments must agree within 0.5% T.

(3) Spectrophotometers. Calibration curves are prepared with certified cyanmethemoglobin standards in the concentration of 5, 10, 15, and 20 g per dl. Each standard is run in triplicate and the % T must agree within ± 0.5%. The curve is plotted and checked daily with one of the hemoglobin standards. Any change in reagent, pipets or spectrophotometers requires preparation of new standard curves.

c. Sources of Error. Regardless of statistical methods, the maximum allowable error for a hemoglobin must not exceed 0.5 g. Prevention of the following errors maintains good quality control.

(1) Instrument Errors:

(a) Inaccurate sample pipet calibration.

(b) Improper calibration of diluent dispenser including volumetric pipets, self-filling pipets, and automatic dilutors.

(c) Failure of automatic dilutors to maintain calibration caused by insecure volume adjustment, dirt in the valves, and bubbles in the chamber.

(d) Colorimeter and spectrophotometer problems including precalibration, obsolete calibration, incomplete calibration (satisfactory at one end of the curve—not at the other), inadequate length of the scale, unstable electrical components, unmatched cuvets, dirty cuvets, and poor standard (evaporation and deterioration).

(2) Personal Errors:

(a) Incomplete mixing of the sample.

(b) Inaccurate and careless pipetting.

(c) Inaccurate adjustment of the colorimeter.

(d) Inaccurate observation of machine readings (carelessness, fatigue, and bias).

(e) Inaccurate conversion of absorbance or % T to g per dl of hemoglobin.

(f) Recording errors.

(g) Transcription errors.

7-6. Control in Cell Counting (Hemacytometer):

a. Sources of Error. There are many variables contained in these methods.

(1) Instrument Errors:

(a) Inaccurate diluting pipets.

(b) Inaccurate counting chamber.

(c) Contaminated diluent fluid.

(d) Irregular cell distribution (field error).

(2) Personal Errors:

(a) Insufficient mixing of the sample.

(b) Inaccurate pipetting of the sample and diluent.

(c) Insufficient or excessive shaking of the sample pipet.

(d) Inaccurate filling of the counting chamber.

(e) Inaccurate counting (fatigue, bias, etc.).

(3) Limits of Precision.

(a) RBC (at 5,000,000) accurate to ±16%.

(b) WBC (7,000) to ±21% (Berksun, Magath, and Hurn).

b. Minimizing Errors:

(1) Sampling in duplicate pipets.

(2) Filling duplicate chambers.

(3) Doubling the number of cells to be counted.

(4) Calculate standard deviation by the duplicate analysis method.

(a) Run duplicates on 20 routine specimens.

(b) Sum the differences between the duplicates of the 20 specimens.

(c) Calculate S.D. using the following formula:

$$\text{S.D.} = \sqrt{\frac{\text{Sum (differences)}^2}{2 \times \text{number of pairs}}}$$

(d) Cell counts must be maintained within the standard deviation.

7-7. Control in Electronic Counting:

a. Controls. Controls for RBCs and WBCs are available commercially. Statistical analysis must be performed to establish confidence limits of the instruments.

b. Calibration. These instruments must be calibrated and maintained according to the manufacturer's instructions.

c. Sources of Error. Electronic cell counting can be done with much greater precision, but possible sources of error are even more numerous.

(1) Instrument Errors:

(a) Inaccurate pipet calibration.

(b) Inaccurate calibration of diluent dispenser.

(c) Inaccurate calibration of current and threshold settings.

(d) Electronic malfunction.

(e) Defective or dirty manometer.

(f) Leaking seals in the glass components.

(g) Partially plugged aperture.

(h) Dirty glassware.

(i) Foaming (detergent residues or saponin).

(j) Loss of cells on standing.

(k) Incomplete hemolysis (for WBC).

(l) Contaminated diluting fluid.

(m) Excessive number of large platelets.

(2) Personal Errors:

(a) Inaccurate pipetting.

(b) Incomplete mixing of original sample and of diluted sample.

(c) Failure to adjust the instrument controls properly.

(d) Errors in recording counts.

(e) Errors in reading correction factors.

(f) Failure to correct WBC counts above 10,000.

(g) Failure to correct cell RBC counts.

7-8. Control in Hematocrit Determinations:

a. Standardization of the Method. Controls to validate hematocrit determinations are available from commercial sources. It is important to use capillary tubes of the best quality to minimize errors due to variation in the diameter of the tubing. The tubes must be adequately sealed to prevent loss of blood during centrifugation. Centrifugation speed and time must be able to produce a constant volume of cell pack. Hematocrit readers must provide accurate positioning of the blood column and have an easily read and accurate scale.

b. Sources of Error:

(1) Instrument Errors:

(a) Variation in caliber of capillary tubes.

(b) Inaccurately calibrated macrohematocrit tubes.

(c) Insufficient centrifuge speed—failure to check brushes routinely.

(d) Insufficient running time.

(e) Leaking tube seals.

(f) Poorly designed microtube reader.

(2) Personal Errors:

(a) Incomplete mixing of the sample.

(b) Incomplete sealing of tubes.

(c) Reading errors.
(d) Transcription errors.

7-9. Control in Differential Counts. Reading differential slides requires a great deal of experience and technical skill. All technicians should have sufficient training to be competent in reading differential smears. However, in certain instances these slides must be reviewed by another technician, laboratory supervisor, or pathologist. The instances are:

a. Counts below 4,000 cells per cu mm and above 15,000 cells per cu mm.

b. A lymphocyte count above 40% for adult and 60% for a child.

c. The observation of abnormal or atypical cells.

d. The observation of any nucleated erythrocytes.

e. Platelets which appear increased, decreased, or abnormal.

7-10. Control in Coagulation Studies:

a. Controls. Citrated plasma controls are available from the Federal Supply Catalog. Controls are run with each applicable test and statistical analysis is performed. To adequately control these procedures all specimens must be performed in duplicate or even triplicate. The differences between these replicate analyses must be within the confidence limits of the laboratory.

b. Sources of Error:

(1) Glassware. Glassware must be clean and of standard size. Coagulation glassware should never be used for other tests. All pipets must be accurate and clean. Precautions must be taken when pipetting to avoid cross-contamination of specimens or reagents.

(2) Lighting. Insufficient lighting can cause a failure to observe an endpoint (clot formation).

(3) Incubation. The water bath temperature must be controlled to 37° C. Variation of this temperature produces invalid results.

(4) Clean Venipuncture. Venipunctures must be nontraumatic. Contamination with tissue thromboplastin leads to erroneous results.

(5) Anticoagulants. Blood must be placed in proper amounts of the desired anticoagulant immediately and mixed well.

(6) Delay in Testing. Testing must be performed as soon as possible after receiving the specimen.

(7) Accurate Timing. Starting the stopwatch must be coordinated with the addition of the final reagent or ingredient, and stopping the watch, with appearance of the fibrin clot.

(8) Reagents. Reagents must be fresh and stable. Expiration dates must be checked.

(9) Technical Skill. A great deal of skill is required in the performance of coagulation tests. Directions must be followed explicitly.

c. Fibrometer. The Fibrometer has eliminated many variables in coagulation testing. It is recommended that Fibrometer methods be employed if possible.

7-11. Control in Other Tests. Many procedures are outlined in chapter 5 which are not performed as frequently as CBCs. However, quality control can still be established in these procedures. For example, abnormal and normal specimens can be analyzed in the same manner as the patient's specimen. This will indicate if a particular stain or reagent is detecting what it is designed to detect. In some cases, controls can be difficult to obtain. For example, controls for hemoglobin electrophoresis should include AS, SS, SC, and AA hemoglobins; however, all but AA hemoglobin can be difficult to obtain. Larger laboratories may be the source of supply for these controls.

7-12. Summary. An experienced technician, well-informed in quality control principles and procedures, should direct and monitor the quality control program of any given clinical laboratory. An effective quality control program for clinical hematology requires attention, interest, and consistency of application on the part of all assigned personnel.

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The Adjutant General

SUMMARY OF REVISED, DELETED, OR ADDED MATERIAL

Revision encompasses a rearrangement and additions to the subject material. The introductory chapter consists of the basic concepts of hematology. The chapter on blood cell counting chambers and dilution pipets is enlarged to include equipment employed in hematology with emphasis on automation. The chapter covering the collection and processing of blood specimens includes bone marrow aspiration and processing. The morphology and maturation of erythrocytes, leukocytes, and thrombocytes are included in one chapter. The methodology of hematology is combined into another chapter. The chapter on blood coagulation studies is enlarged to include all pertinent tests aiding the study of blood coagulation. A chapter is included to present quality control aspects in hematology.

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GLOSSARY

Agranulocyte: A leukocyte without definite cytoplasmic granules.

Agranulocytosis: Complete or nearly complete absence of the granular leukocytes from the blood and bone marrow.

Aleukemic Leukemia: A fatal condition of the blood-forming tissues, characterized by marked proliferation of immature cells in the bone marrow, without their presence, in any great numbers, in the blood stream.

Anemia: A condition in which the blood is deficient in quantity or quality of erythrocytes.

Anisocytosis: Variation in size of the erythrocytes.

Anomaly: Abnormality.

Anoxemia: Lack of normal proportion of oxygen in the blood.

Antecubital Space: The area on the forearm frontal to the elbow.

Anticoagulant: A substance that prevents the coagulation of blood. Commonly used ones are potassium oxalate, sodium oxalate, sodium citrate, EDTA, and heparin.

Aplasia: Incomplete or defective blood development; cessation of blood cell formation.

Aplastic Anemia: Anemia characterized by incomplete or defective blood development.

Asynchronous: Uncoordinated development.

Azurophilic Granule: Rounded, discrete, reddish-purple granule, smaller than the granules of neutrophils; 0–10 are common in lymphocytes, and they are very numerous, and smaller, in the cytoplasm of monocytes.

Band Form: In the Schilling classification, a neutrophil with the nucleus unsegmented and ribbonlike; also stab, staff, nonfilamented.

Basket Cell: A degenerated primitive cell which has ruptured and in which the cell nucleus appears as a pale staining smear without prescribed form or shape.

Basopenia: An abnormal decrease in the number of basophils.

Basophil: A granular leukocyte, the granules of which have an affinity for the basic dye of Wright's stain (methylene blue). The granules are large, irregular and blue-black in color.

Basophilia: An abnormal increase in the number of basophils.

Basophilic: Staining readily with basic dyes, for example, blue with Romanovsky type stains.

Binary Fission: Simple cell division.

Bleeding Time: The time required for a small standardized wound, made in the capillary bed of the finger or ear lobe, to stop bleeding.

Blood Dyscrasia: A disease of the blood or blood-forming organs.

Buffy Coat: The layer of leukocytes that collects immediately above the erythrocytes in sedimented or centrifuged whole blood.

Cabot's Rings: Lines in the form of loops or figures-of-eight seen in erythrocytes in severe anemias.

Centriole: A minute cell organoid within the centrosome.

Centrosome: An area of condensed cytoplasm active in mitosis.

Chemotaxis: The phenomenon of movement of leukocytes caused by a chemical influence.

Chromatin: The more stainable portion of the cell nucleus, contains genetic materials.

Clot Retraction: The rate and degree of contraction of the blood clot.

Coagulation Time: The time required for venous blood, in the absence of all tissue factors, to clot in glass tubes under controlled conditions.

Cocatalyst: A substance that works in tandem with another group of chemicals to accelerate a reaction velocity without being used up in the reaction.

Color Index: The ratio between the amount of hemoglobin and the number of red blood cells.

Complete Blood Count: A hematology study which consists of a red cell count, white cell count, hematocrit, hemoglobin, and blood smear study including differential white cell count.

Congenital: Born with a person; existing at or before birth.

Cooley's Anemia (Mediterranean Disease or Thalassemia): A chronic progressive anemia commencing early in life and characterized by many normoblasts in the blood, unusual facies, splenomegaly and familial and racial incidence. Target type red blood cells are often present in the peripheral blood.

Crenation: The scalloped or notched appearance of the periphery of erythrocytes found when the cells are suspended in a hypertonic solution. Also found in smears, caused by dirty glassware, slow drying and poor smearing technique.

Cytoplasm: Protoplasm of a cell excluding the nucleus.

DNA: Deoxyribonucleic acid.

Differential Count: An enumeration of the types of white blood cells seen on a stained blood smear.

Discrete: Separate.

Dyscrasia: Abnormality.

Ecchymosis: Subcutaneous extravasation of blood covering a large area.

Endothelial Leukocyte: Monocyte.

Eosinopenia: An abnormal decrease in eosinophils.

Eosinophil: A granular leukocyte, the granules of which have an affinity for the acid dye of Wright's stain (eosin). The granules are large, round, uniform in size, red-orange in color and are shiny and refractile.

Eosinophilia: A relative or absolute leukocytosis in which the main increase is in eosinophil.

Eosinophilic: Readily stained with eosin; red-orange stain.

Epigastric: Pertaining to the upper middle portion of the abdomen.

Erythremia: A disease marked by persistent polycythemia and increased blood volume; also polycythemia vera.

Erythrocyte: Red blood cell.

Erythrocytosis: An increase in the total number of erythrocytes.

Erythrogenic: Producing erythrocytes.

Erythroleukemia: An abnormal condition characterized by proliferation of erythroblastic and myeloblastic cells.

Erythropenia: A decrease in the number of red cells in the blood.

Erythropoiesis: The production of erythrocytes.

Etiology: The theory of the causation of a disease.

Extravascular: Occurring outside of the blood vessels.

Extrinsic: Originating outside of the particular area.

Fibril: A microscopic filament often composed of fibrin.

Fibrin: The end product of the clotting mechanism which forms a network of fibers that enmesh the formed elements of blood.

Fibrinogen: The precursor of fibrin which is present normally in the plasma and produced by the liver.

Fragility Test (Osmotic): A test devised to measure the resistance of the erythrocytes to break down (hemolyze) when subjected to varying concentrations of hypotonic salt solutions.

Fulminating: Sudden and severe.

Golgi Apparatus: A meshwork of lipid containing fibrils within the cytoplasmic portion of a cell.

Granulocyte: A white blood cell that contains specific cytoplasmic granules (neutrophils, eosinophils, and basophils); these granules are peroxidase positive.

Granulocytosis: The presence of increased numbers of granulocytes in the blood.

Granulocytopenia (Granulopenia): A decrease in the number of granulocytes in the blood.

Granulopoiesis: The production of granulocytes.

Hemacytometer: A calibrated chamber in which blood cells are counted.

Hematin: A brown or blue-black amorphous iron substance which unites with globin and forms hemoglobin.

Hematocrit: The packed cell volume (PCV) of red blood cells obtained by centrifugation of a blood specimen in a hematocrit tube.

Hematology: The branch of medicine that deals with the study of blood cells, blood-producing organs and the manner in which these cells and organs are affected in disease.

Hematoma: Subcutaneous effusion of blood with resulting swelling, pain, and discoloration, forming a tumorlike mass.

Hematopoietic (Hemopoietic): Blood forming.

Hemoglobin: The coloring matter of the red blood cells. A complex iron-bearing pigment which carries oxygen and carbon dioxide.

Hemoglobinuria: The presence of free hemoglobin in the urine.

Hemogram: The blood picture.

Hemolysis: The dissolution or dissolving of the erythrocytes.

Hemolytic Anemia: That type of anemia characterized by excessive intravascular destruction of red cells.

Hemophilia: A hereditary disease characterized by a prolonged coagulation time and repeated hemorrhages, occurring only in males and transmitted only by females and affected males. The cause is a deficiency in a plasma factor (antihemophilic globulin or thromboplastinogen) resulting in a defect in thromboplastic activity.

Hemoptysis: The spitting of blood; coughing up blood.

Hemostasis: The checking of the flow of blood, especially from a vessel.

Hepatic: Originating from the liver.

Heterozygous: Derived from germ cells unlike in respect to one or more factors.

Homeostasis: Stability in normal body states.

Homozygous: Derived from germ cells which are alike.

Howell-Jolly Bodies: Small basophilic particles sometimes found in erythrocytes, remnants of nuclear material.

Hygroscopic: Readily taking up and retaining water.

Hyperplasia: An increase in cell formation.

Hypertonic: Greater than isotonic concentration.

Hypertrophy: Enlargement of an organ or part due to increase in the size of the constituent cells.

Hypochromia: A decrease in color of the erythrocytes, hence a decrease in their hemoglobin content.

Hypoplasia: A decrease in cell formation.

Hypotonic: Less than isotonic concentration.

Idiopathic: Disease of unknown cause.

Inclusion: Usually lifeless, an accumulation of fats, proteins, crystals, pigments or secretory granules within a cell cytoplasm.

Inhibitor: A substance, directed against a coagulation factor or factors, which interferes with the coagulation process.

Intravascular: Occurring within the blood vessels.

Intrinsic: Situated within the particular part.

In Vitro: Within a test tube (glass, etc.).

In Vivo: Within the living organism, as in life.

Isotonic: Solutions with the same osmotic pressure.

Jaundice: Yellowness of the skin and eyes due to the presence of blood pigments in the blood; follows excessive destruction of the blood, obstruction of the bile passage, diffuse liver disease, certain infections, toxic chemical agents and drugs.

Juvenile Cell: In the Schilling classification, the cell between the myelocyte and band forms; also metamyelocyte.

Karyolysis: Apparent destruction of the nucleus of a cell.

Karyorrhexis: Fragmentation of the nucleus; a degenerative process usually followed by karyolysis.

L.E. Cell: A large segmented neutrophil or eosinophil that contains ingested autolyzed nuclear fragments in its cytoplasm.

Leukemia: An ultimately fatal disease of the blood-forming organs characterized by increased numbers of leukocytes and associated anemia.

Leukemoid Crisis or Reaction: A temporary appearance of immature leukocytes in the blood stream, with a marked increase in the total white count. In the laboratory sometimes temporarily indistinguishable from leukemia.

Leukocyte: White blood cell.

Leukocytosis: An increase in leukocytes in the blood.

Leukopenia: A reduction in the number of leukocytes in the blood.

Leukopoiesis: Leukocyte formation.

Lymphoblast: The parent cell of the lymphocytic series.

Lymphocyte: A white blood cell having a round or oval nucleus and sky blue cytoplasm. The nuclear chromatin is densely clumped but separated by many clear areas giving a "hill and valley" effect. A few red-purple (azurophilic) granules may be present in the cytoplasm.

Lymphocytosis: A relative or absolute increase in the number of circulating lymphocytes.

Lymphopenia: An abnormal decrease in the number of lymphocytes.

Lysis: Destruction by a specific agent.

Macrocyte: An erythrocyte larger than normal.

Macrocytosis: An increase in the number of macrocytes.

Mast Cell: A basophil or a true tissue cell.

Maturation Factor: A substance which will cause cells to ripen and come to maturity.

Mean Corpuscular Hemoglobin (MCH): The average amount of hemoglobin in the red blood cell.

Mean Corpuscular Hemoglobin Concentration (MCHC): The average percent hemoglobin saturation in the red blood cell.

Mean Corpuscular Volume (MCV): The volume of the average red blood cell.

Megakaryoblast: The parent cell of the megakaryocytic series.

Megakaryocyte: An extremely large cell with an irregularly lobed, ring- or doughnut-shaped nucleus which stains blue-purple. The cytoplasm is abundant, light blue and is packed with fine azurophilic granules. This cell gives rise to thrombocytes.

Megaloblast: The type of red cell precursor found in pernicious anemia. This differs from the normal erythrocyte precursor (normoblast) in that the megaloblast is larger and the nuclear chromatin has a fine meshwork or scroll design.

M:E Ratio: The ratio of myeloid to erythroid cells in the bone marrow.

Mesentery: The fold of peritoneum which attaches the intestine to the posterior abdominal wall.

Metamyelocyte: Juvenile cell of Schilling.

Metarubricyte: An erythrocyte with a pyknotic, contracted nucleus. Also called orthochromatophilic normoblast.

Methemoglobin: A spectroscopically detected compound of hemoglobin found in nitrobenzol, and other poisonings. The blood is a chocolate brown color to the eye.

Microcyte: An erythrocyte smaller than normal.

Microcytosis: An increase in the number of microcytes.

Micron: One-thousandth of a millimeter, the common unit of microscopic measure.

Mitochondria: Granular components of a cell cytoplasm active in oxidative processes.

Mitosis: A series of changes through which the nucleus passes in indirect cell division. A tissue showing many cells in mitosis indicates rapid growth of that tissue.

Monoblast: The parent cell of the monocytic series.

Monocyte: A large white blood cell with a pale blue-gray cytoplasm containing fine azurophilic granules. The nucleus is spongy and lobulated.

Monocytosis: A relative or absolute increase in the number of circulating monocytes.

Mucosa: Mucous membrane.

Myeloblast: The parent cell of the granulocytic or myelocytic series.

Myelocyte: The stage in development of the granulocytic series which is characterized by the first appearance of specific granules (eosinophilic, neutrophilic or basophilic) and a round nucleus.

Myeloid Cells: The granular leukocytes and their stem cells.

Myelopoiesis: Formation of bone marrow and the blood cells that originate in the bone marrow.

Myeloproliferative: Rapid production of bone marrow constituents.

Necrosis: The death of a circumscribed portion of tissue. Simple necrosis is degeneration of the cytoplasm and nucleus without change in the gross appearance of the tissue.

Neutropenia: A decrease in the number of neutrophils in the blood.

Neutrophil (Polymorphonuclear Neutrophil or Segmented Neutrophil): A granulocyte having fine neutrophilic (pink-violet) granules in the cytoplasm. The nucleus is divided into two or more lobes; each lobe is usually connected by a filament.

Neutrophilia: An increase in neutrophils.

Normoblast: The nucleated precursor of the normal red blood cell. Also called a rubriblast.

Normocyte (Erythrocyte): A red blood cell of normal size.

NRBC: Nucleated red cell, usually a metarubricyte when seen in the peripheral blood smear.

Nucleolus: An intranuclear pale blue body, surrounded by a dense condensation of chromatin.

Occult Blood: The presence of blood which cannot be detected except by special chemical tests.

Oligochromemia: A decrease in hemoglobin.

Oligocythemia: A decrease in the number of erythrocytes.

Organoid: Structures present in cells resembling organs.

Ovalocyte: An elliptical erythrocyte.

Oxyhemoglobin: The bright red hemoglobin that is loosely combined with oxygen and found in arterial blood.

Pancytopenia: A reduction in all three formed elements of the blood, namely, the erythrocytes, leukocytes and thrombocytes.

Pathologic Increase (Or Decrease): Due to abnormal function or disease, as contrasted to physiological (due to normal body function).

Pernicious Anemia: A chronic, macrocytic anemia caused by a defect in production of "intrinsic factor" by the stomach. There is accompanying megaloblastic erythropoiesis, poikilocytosis, granulocytic hypersegmentation, achlorhydria, and neurological disturbances.

Petechiae: Small spots on the skin formed by subcutaneous effusion of blood (also purpura and ecchymoses).

Phagocytosis: The destruction of organisms and extraneous matter by a process of envelopment and absorption.

Plasma: The fluid portion of the blood composed of serum and fibrinogen, obtained when an anticoagulant is used.

Plasma Cell: A lymphocyte-like cell with an eccentrically placed deep-staining nucleus. The nuclear chromatin is distributed in a "wheel-spoke" fashion. The cytoplasm is deep blue with a lighter halo about the nucleus.

Platelet: Thrombocyte.

Poikilocyte: A red blood cell having an abnormal shape (pear-shape, sickle-shape, etc.).

Poikilocytosis: Increased number of abnormally shaped erythrocytes.

Polychromasia: Diffuse basophilia of the erythrocytes.

Polychromatophilia: The presence in the stained blood smear of immature, nonnucleated, bluish-staining red blood cells.

Polycythemia: An increase in the total number of erythrocytes. (See erythremia.)

Precursor: A substance from which another substance is formed.

Promyelocyte: The precursor of the myelocyte having nonspecific azurophilic (red-purple) cytoplasmic granules.

Prorubricyte: The second stage of development of the red cell.

Prothrombin: The inactive precursor of thrombin which is formed in the liver and present normally in the plasma. Its formation depends upon adequate vitamin K.

Punctate Basophilia: Small basophilic aggregates in the erythrocytes that stain blue with the basic dye of Wright's stain; also basophilic stippling.

Purpura: Small spots on the skin formed by subcutaneous effusion of blood.

Pyknosis: A condensation and reduction in size of the cell and its nucleus.

Reduced Hemoglobin: A combination of hemoglobin and carbon dioxide which is found in venous blood.

Reticulocyte: A red blood cell showing a reticulum or network when stained with vital dyes (for example, brilliant cresyl blue). The stage between the nucleated red cell and the mature erythrocyte.

Reticulocytosis: An increase above normal values of reticulocytes in peripheral blood.

RNA: Ribonucleic acid.

Rouleaux Formation: The arrangement of red cells with their flat surfaces facing, in which they appear as figures resembling stacks of coins.

Rubricyte: Polychromatophilic normoblast.

Sedimentation Rate, Erythrocyte (ESR): The rate at which red cells will settle out in their own plasma in a given time under controlled conditions.

Serum: The fluid portion of the blood, after clot formation.

Shift to the Left: A term used to designate that condition in which the immature forms of the neutrophils are increased above their normal number.

Shift to the Right: Increase in mature, pyknotic, and hypersegmented neutrophils.

Sickle Cell: A sickle- or crescent-shaped erythrocyte.

Sickle Cell Anemia: This is a hereditary and familial form of chronic, hemolytic anemia essentially peculiar to Negroes. It is characterized clinically by symptoms of anemia, joint pains, leg ulcers, acute attacks of abdominal pain and is distinguished hematologically by the presence of distinct hemoglobin, peculiar sickle-shaped and oat-shaped red corpuscles, and signs of excessive blood destruction and active blood formation.

Smudge Cell: A ruptured white cell; also basket cell, or degenerated cell.

Spherocyte: A red blood cell which is more spherical, smaller, darker and more fragile than normal.

Stasis: A stoppage of blood flow.

Supravital Stain: A stain of low toxicity which will not cause death to living cells or tissues.

Synchronous: Occurring at the same time and in a regular pattern.

Target Cell (Leptocyte): An abnormal, thin erythrocyte characteristic of Cooley's or Mediterranean anemia.

Triturate: To grind together.

Thrombin: This is an enzyme formed from prothrombin which converts fibrinogen to fibrin. This is not present in circulating blood.

Thrombocyte: A blood platelet.

Thrombocytopenia: A decrease in blood platelets; also thrombopenia.

Thrombocytosis: An increase in blood platelets.

Thromboplastin: The substance that initiates the process of blood clotting. It is released from injured tissue and/or formed by the disintegration of platelets in combination with several plasma factors.

Thrombopoiesis: The production of thrombocytes.

Thrombosis: Formation of a thrombus, or blood clot.

Vacuole: A space or cavity formed in the protoplasm of a cell.

Venipuncture: The act of puncturing a vein in order to remove a sample of blood.

Viscous Metamorphosis: Friction between molecules resulting in a structural change.

Vitamin K: A vitamin constituent of the normal diet requiring bile salts for absorption. This vitamin is utilized by the liver in the production of prothrombin.

Xanthochromia: A yellowish discoloration, usually associated with spinal fluid.

PREFIXES, SUFFIXES, AND STEMS COMMONLY USED IN HEMATOLOGY

Hematologic Usage	*Meaning*	*Hematologic Usage*	*Meaning*
a- or an-	without	micro-	small
aniso-	unequal	mono-	single
baso-	blue	myelo-	marrow
-blast	primitive form	ortho-	correct or normal
-chrome	color	-osis	state of, condition of, increase of
crena-	a notch		
-crit	to separate	-penia	decrease of
-cyte	cell	phago-	to eat
eosino-	red-orange	-philos	attraction for
erythro-	red	-plasia	a forming or molding
hem- haem- hemato-	blood	-plastic	to form
hyper-	excessive	-poiesis	to make
hypo-	deficient	poikilo-	varied
iso-	equal	poly-	much or many
leuk- leuko-	white	pro-	before
macro-	large	reticulo-	a net
mega-	great or huge	thrombo-	clot
meta-	after or next	-tonos	strain or tension

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